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Original Contribution

v-Ha-RaS ONCOGENE UPREGULATES THE 92-kDA TYPE IV COLLAGENASE (MMP-9) GENE BY INCREASING CELLULAR SUPEROXIDE PRODUCTION AND ACTIVATING NF-κB

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Abstract—Matrix metalloproteinase 9 (MMP-9) degrades basement membrane type IV collagen and is expressed during cellular migration and invasion. Here we show that v-Ha-Ras overexpression in rat kidney epithelial cells (REC) caused upregulation of MMP-9 gene expression in part by increasing cellular oxidant levels. v-Ha-Ras mediated the production of superoxide in Ras-transfected cells, which was associated with upregulated MMP-9 gene expression. Conversely, v-Ha-Ras expression decreased steady-state levels of mRNAs from tissue inhibitor of metalloproteinase 1 (TIMP-1), an inhibitor of MMP-9; plasminogen activator inhibitor 1 (PAI-1), which indirectly activates MMP-9 by increasing plasmin levels; and collagen IV, a substrate of MMP-9 and a major component of basement membrane. Gel mobility shift assays demonstrated that Ras overexpression enhanced NF-κB, but not AP-1 DNA binding to motifs in the MMP-9 gene promoter. The Ras-induced increase in NF-κB DNA binding could be inhibited by treatment with the antioxidants N-acetyl-L-cysteine and glutathione monoester, suggesting that intracellular oxidant levels can mediate MMP-9 transcription. Our findings identify an important role for Ras in the regulation of MMP-9 expression, and suggest that increased superoxide production can upregulate MMP-9 expression and thus contribute to malignant conversion. © 2001 Elsevier Science Inc.

Keywords—Free radicals, Superoxide, Redox, Ras, Extracellular matrix, Metalloproteinase

INTRODUCTION

Matrix metalloproteinases (MMPs) are members of a unique family of zinc-binding endopeptidases that share several features. All MMPs contain a zinc ion at their active site and are inhibited by chelating agents. They share significant sequence homologies and are all secreted in a latent form that becomes activated by partial proteolytic cleavage. Finally, the MMPs can be inhibited by specific tissue inhibitors of metalloproteinases (TIMPs) [1]. MMPs are responsible for degradation of the components of extracellular matrix (ECM). ECM provides not only structural support, but also assists in cell proliferation, adhesion, migration, and tissue morphogenesis [2]. Different MMPs have different substrate specificities, for example, 72 kDa and 92 kDa Gelatinases/type IV collagenases (MMP-2 and MMP-9, respectively) can degrade type IV and V collagens and fibronectin, laminin, and gelatins [3–5]. As expected from the nature of their enzymatic activity, the uncontrolled expression of MMPs has been reported to be associated with many pathological conditions and events such as atherosclerosis [6], tumor metastasis, and rheumatoid arthritis [7–9], whereas their controlled expression is essential for normal processes such as embryogenesis, tissue remodeling, and wound healing.

The expression of MMP-9 activity is controlled at several levels, including gene transcription, latent proenzyme activation, and inhibition of enzymatic activity by TIMP1. After the rat MMP-9 gene cDNA (2,127 bp) was cloned, comparison of the amino acid sequence of the rat MMP-9 to those of the human and

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mouse shows a 74% and 80% homology, respectively [10]. The human MMP-9 is encoded by a 7.7 kb pair gene that spans 13 exons on chromosome 20q [11]. Transcription of the rat MMP-9 gene is regulated by at least 737 bp of 5′ regulatory sequence, which includes two binding sites for AP-1 (−512 to −506, −87 to −81) and one binding site for NF-κB (−569 to −562) [11]. After cleavage of a signal peptide domain of about 20 amino acids, the MMP-9 is first secreted in latent form. Upon activation, the N-terminal propetide domain is cleaved to leave the active form of the MMP-9. The activity of the MMP-9 can be inhibited by endogenous inhibitor TIMP1, as well as by rapid autodegradation of the C-terminal domain.

Matrix metalloproteinase 9 (MMP-9) is of special interest because of its degradative activity against basement membrane type IV collagen and its expression in cells of emigrational or invasive nature during physiological and pathological processes. MMP- 9 is often overexpressed by malignant tumor cells, but its expression is not necessarily coordinated with that of interstitial collagenase and stromelysin-1 [12]. In fact, expression of MMP-9 mRNA correlates well with tumorigenicity and metastatic ability of tumor cells [13]. Several lines of evidence suggest an important role for Ras in the regulation of MMP-9 expression [14]. We hypothesized that Ras might regulate MMP-9 expression by superoxide production, and that suppression of Ras-mediated superoxide production would affect MMP-9 expression.

MATERIALS AND METHODS

Cells and cell culture

The rat kidney epithelial cell line (REC) NRK52E was obtained from ATCC (ATCC, Manassas, VA, USA: CRL-1571) [15]. Cells were maintained in high glucose DMEM medium containing 5% bovine calf serum, 2 mM L-glutamine, and 0.1 mM nonessential amino acids at 37°C in 5% CO_2. Cells were fed three times a week. v-Ha-Ras-transduced REC [16] were also cultured with 400 μg/ml G418. The cells used in our experiments were between passage numbers 10 and 40.

Western blotting analysis

Cells were harvested and lysed by sonication, and then proteins (concentrations quantitated using Bio-Rad [Hercules, CA, USA] protein assay kit) separated by 12.5% SDS-PAGE. The proteins were electrotransferred to nitrocellulose sheets. After blocking in 5% milk for 30 min, the sheets were washed and treated with antibody to pan-Ha-Ras (1:1000; purchased from Oncogene Science, Cambridge, MA, USA). The blots were washed three times for 15 min each with TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0). The blots were then incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:10,000; Sigma Chemical Co., St. Louis, MO, USA) for 1 h at room temperature. The washed blots were then treated with ECL Western blot detection solution (Amersham Life Science, Piscataway, NJ, USA) and exposed to X-ray film.

SOD-inhibitable cytochrome c assay

The production of superoxide was measured by its reduction of cytochrome c [17]. Cells were cultured in 6 well plates. The medium was then removed and cells washed twice in Krebs Ringer buffer. Cytochrome c (100 μM) in Krebs Ringer buffer with catalase (200 U/ml) was added to the wells; samples were removed after 1 h of incubation at 37°C in 5% CO_2. The spectrum of each sample was then taken between 400 and 600 nm. Superoxide production was determined by measuring the peak height at 550 nm over the baseline between 530 and 560 nm. Cytochrome c can be reduced by $O_2^{\bullet -}$ from cyt c (Fe^{3+}) to cyt c (Fe^{2+}), with a rate constant $k = 1.5 \times 10^5$ $M^{-1}s^{-1}$ (pH 7–9.3), and extinction coefficients of cyt c (Fe^{2+}) $\varepsilon_{m550} = 2.99 \times 10^4$ $M^{-1}cm^{-1}$ and cyt c (Fe^{3+}) $\varepsilon_{m550} = 0.89 \times 10^4$ $M^{-1}cm^{-1}$ [18].

Reverse-transcription and polymerase chain reaction (RT-PCR)

Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method, using Tri-ZOL reagent (GIBCO, Grand Island, NY, USA); the concentration of RNA was determined spectrophotometrically at 260 nm. To avoid DNA contamination, 10 μg of total RNA were incubated with 2 μl reaction buffer, 2 units DNase (Promega, Madison, WI, USA) and RNase-free H_2O for 30 min at 37°, then samples were heated at 65°C for 10 min to inactivate DNase. The MMP-9 upstream and downstream primers were 5′-GCCATTGCTGATATCCA-3′ and 5′-GCCTTGTCTTGGTACTGA-3′, respectively, and encompassed bases 1576–1592 and 1987–2004. The predicted product was 446 bp in length. The RT-PCR reactions were carried out using one-step RT-PCR kit (Qiagen, Santa Clarita, CA) in 50 μl reaction solution containing 1 μg total RNA, 10 μl of 10X PCR buffer, 2 μl of 10 mM dNTP, 1 μM of sense and antisense primers, 8 units of RNase inhibitor (Promega), and 2 μl enzyme mixture. RT-PCR reaction solution was incubated at 50°C for 30 min and denatured at 95°C for 15 min, followed by 30 cycles at 94°C for 1

min, 54°C for 40 s, 72°C for 40 s. The final extension step was 72°C for 7 min. The 446 bp PCR products of MMP-9 were cloned into the pCR2.1 vector and transformed into competent cells as described by the manufacturer (TA Cloning Kit, Invitrogen, Carlsbad, CA, USA). DNA was isolated (Promega) and sequenced by the DNA Core at the University of Iowa.

Northern blot analysis

Total RNA was isolated by the acid guanidinium thiocyanate phenol-chloroform extraction method, and then quantitated by spectrophotometry. Eighteen μg RNA were electrophoresed on a 1.5% agarose gel. RNA was transferred to a nylon membrane, the membrane was then preincubated with preincubation buffer (50% formamide, 5× SSC, 10% dextran sulfate, 1× Denhardt's buffer, 1% SDS, and 150 μg/ml denatured salmon sperm ssDNA) and hybridized overnight to a ^{32}P-labeled probe made from random prime labeling of target gene cDNA. Following hybridization, the membrane was washed in 2× SSC, 0.5% SDS solution twice for 15 min each at room temperature and then washed in 0.1× SSC, 0.5% SDS solution twice for 15 min each at 65°C. The membrane was wrapped in plastic wrap and exposed to X-ray film at −80°C overnight. 28S RNA was used to visualize the amounts of RNA loading. When the membrane was used with a second probe, the previous probe was removed by boiling the membrane in 0.1% SDS for 2 × 15 min.

Zymographic analysis

Conditioned medium was collected, then concentrated 10 times by centrifuging at 6500 rpm for 2 h using Centricon centrifugal filter devices (Millipore Corp., Bedford, MA, USA). To determine the gelatinolytic activity, 1 mg/ml gelatin substrate was prepolymerized in the 10% SDS-polyacrylamide gel. Ten μl of concentrated conditioned serum free media were mixed with 10 μl sample buffer and were separated electrophoretically at 120 V for 1–1.5 h. The gel was then washed in 2.5% Triton X-100 and 50 mM Tris/HCl, pH 8.0 for 2 × 30 min and incubated overnight in a developing buffer containing 50 mM Tris/HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 150 mM NaCl at 37 °C. The gel was stained with 0.25% Coomassie Blue in 25% isopropanol/10% acetic acid for 30 min and destained in buffer containing 10% methanol and 10% acetic acid until the wash remained clear. Gelatinolytic bands appeared as clear zones against the blue background.

Gel mobility shift assays

Nuclear protein was extracted by the following procedure. Cells were collected when they reached 80% confluence and rinsed with PBS buffer. Cells were then harvested by scraping in the presence of ice-cold buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl) and incubated on ice for 20 min. The cells were lysed with a dounce homogenizer and the nuclei were pelleted by centrifuging at 5000 rpm for 30 s twice at 4°C with subsequent removal of supernatant. The nuclear pellets were respended in ice-cold buffer C (20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF) and placed on ice for 15 min. The suspensions were centrifuged and the supernatants were removed and diluted with ice-cold buffer D (20 mM HEPES, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF). The protein concentrations were determined and nuclear proteins were stored at −80°C.

The DNA probes were double-strand end-labeled by {α^{32}P}dATP in a fill-in reaction of the 5′ overhangs with Klenow DNA polymerase. DNA and protein binding assays were performed by incubating 5 μg of nuclear protein with {α^{32}P}dATP probe DNA (5 μl probe from the above) in the presence of 1 μg poly dIdC (Pharmacia Inc., Piscataway, NJ, USA) and 1 × gel shift buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol) at room temperature for 15 min. Free and bound probes were separated by polyacrylamide gel electrophoresis in a 5% native polyacrylamide gel and run at 35 mA for 40 min in 1 × TBE buffer. For gel supershift experiments, nuclear proteins were preincubated with 0.5 μg anti-transcription factor antibody for 30 min at room temperature and then incubated with ^{32}P-labeled probe for 20 min. The gels were wrapped in plastic wrap and exposed to X-ray film overnight at −80°C.

RESULTS

Rat kidney epithelial cells stably transduced with a constitutively active isoform of oncogene v-Ha-Ras produced large amounts of superoxide

To establish that the v-Ha-Ras stable transfected cells overexpressed the Ras protein, Western blotting analysis was performed with a pan-Ha-Ras antibody as described in Materials and Methods. As shown in Fig. 1A, the transfected cells showed significantly higher amounts of Ha-Ras protein than their parent and Neo control counterparts. Clones U12 and U23 showed the highest levels of Ras expression, while clone W13 displayed Ras protein levels higher than parent and Neo controls, but not as high as U12 and U23.

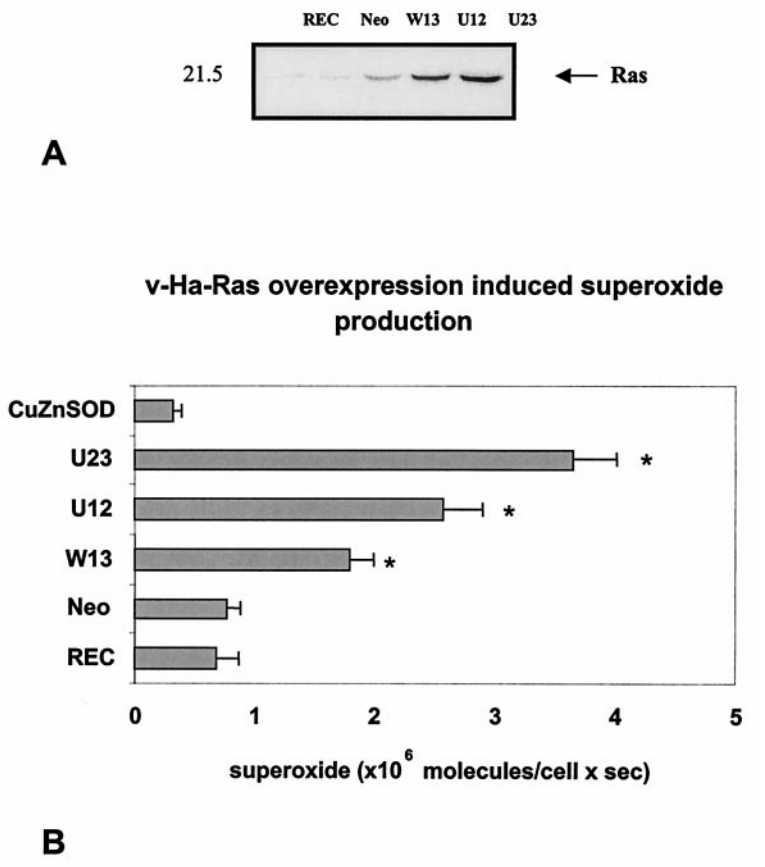

Fig. 1. v-Ha-Ras overexpression induced superoxide production in rat kidney epithelial cells. (A) Western blot analysis of v-Ha-Ras-transfected REC cells showing relative levels of Ras protein. (B) Cellular superoxide levels in Ras-transfected cells as determined by the SOD-inhibitable cytochrome c assay. Cells were washed, then incubated with 100 μM cytochrome c in Krebs Ringer buffer at 37°C, 5% CO_2 for 1 h. Samples were scanned from wavelength 400 nm to 600 nm; the 550 nm peak height was measured and cell numbers were counted after the assay. Data were calculated and expressed as the rate of superoxide production. Means were calculated from three experiments and error bars represent standard deviations. *Indicates significantly different from REC or Neo at the $p < .05$ levels. Data indicate that Ras-transduced cells had increased levels of superoxide radical. U23 was further treated with CuZnSOD (200 U/ml final) added into the cytochrome c solution during the measurement.

Much recent evidence has demonstrated that Ras-transformed cells can stimulate cellular superoxide production [19,20]. To test if v-Ha-Ras-transduced REC cells produce more superoxide, we used Fe(III) cytochrome c to monitor the amount of superoxide production from the cells. The oxidized cytochrome c can be reduced by one electron with superoxide and the amount of reduced cytochrome c monitored at 550 nm. As mentioned in Materials and Methods, cytochrome c can be reduced by $O_2^{\bullet -}$ from cyt c (Fe^{3+}) to cyt c (Fe^{2+}), with a rate constant $k = 1.5 \times 10^5\ M^{-1}s^{-1}$ (pH 7–9.3), and extinction coefficients of cyt c (Fe^{2+}) $\varepsilon_{m550} = 2.99 \times 10^4\ M^{-1}cm^{-1}$ and cyt c (Fe^{3+}) $\varepsilon_{m550} = 0.89 \times 10^4\ M^{-1}cm^{-1}$[18]. Figure 1B shows that v-Ha-Ras-transduced cells U12 and U23 generated large amounts of superoxide, up to 5-fold more than REC or Neo cells. The addition of exogenous CuZnSOD protein totally inhibited the increased superoxide levels seen in U23 cells, which demonstrated that the 550 nm peak was specifically due to the superoxide production from the cells.

v-Ha-Ras expression increased MMP-9 gene expression

Upregulation of 92-kDa type IV collagenase gene expression is associated with tumor cell invasiveness [21]. To determine whether the expression of MMP-9 gene expression was affected by Ras overexpression, we performed RT-PCR analysis with primers specific to rat MMP-9 cDNA. The results of this analysis, shown in Fig. 2, indicate that MMP-9 mRNA was expressed at higher levels in the Ras-transfected cells than in parent REC cells or Neo controls. As a control for the reverse transcription and polymerase chain reaction, glyceraldehyde-3-phosphate dehydrogenase was also amplified and no differences were seen. Although this assay was only semi-quantitative, there was a positive correlation be-

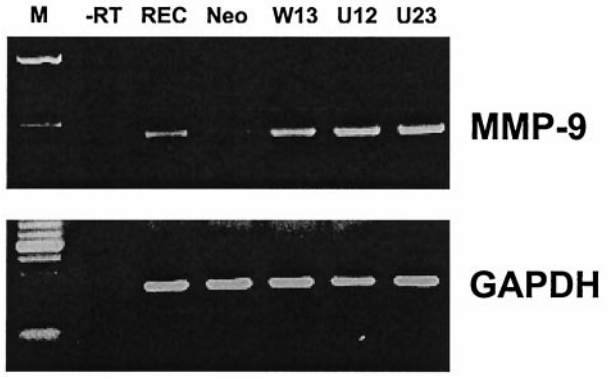

Fig. 2. Steady-state levels of MMP-9 mRNA were upregulated by v-Ha-Ras overexpression as determined by RT-PCR. One μg of total RNA from each cell population was reverse-transcribed with MMLV reverse transcriptase, and the resulting cDNAs were then amplified with primers specific for rat MMP-9 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR products were resolved on a 1% agarose gel and visualized with ethidium bromide. PCR products were sequenced and BLAST searched to verify their identity as rat MMP-9 cDNA. The results shown are representative of two replicate experiments. Lane designations: M = molecular size standards; −RT = negative control without reverse transcriptase.

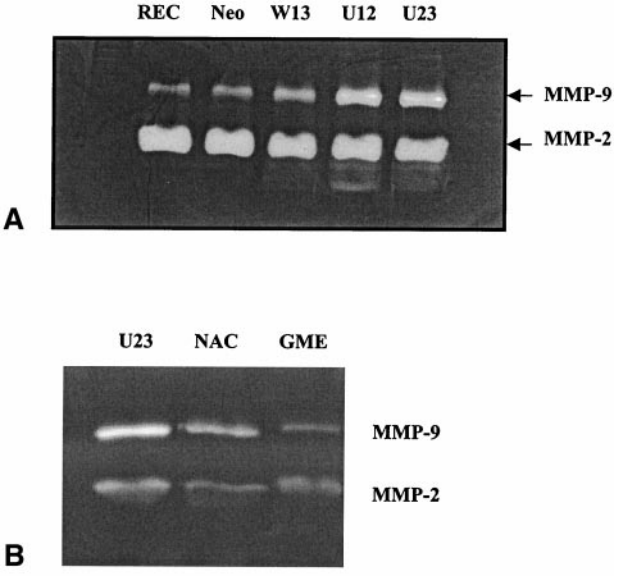

Fig. 3. 92-kDa type IV collagenase (MMP-9) was secreted at higher levels in v-Ha-Ras overexpressing cells. (A) Zymographic analysis for gelatinolytic activity of 92-kDa type IV collagenase showed higher enzyme activity in v-Ha-Ras-transfected cells compared to parent REC cells and Neo controls. Conditioned media were collected from cells at 48 h after replenishment. (B) MMP-9 secretion was attenuated by the treatment of U23 cells with antioxidants. Conditioned media were collected from cells at 48 h after the treatment of 5 mM N-acetyl cysteine (NAC), or 4 mM glutathione monoester (GME). After concentrating, the media was separated in a 7.5% SDS-polyacrylamide gel containing 0.1% (w/v) gelatin. The gel was incubated at room temperature for 2 h in the presence of 2.5% Triton X-100 and subsequently at 37°C overnight in a buffer containing 10 mM $CaCl_2$, 0.15 M NaCl, and 50 mM Tris (pH 7.5). The gel was then stained for protein with 0.25% Coomassie. Proteolysis was detected as a white zone in a dark field. MMP-9 activity was greatly increased in U12 and U23.

tween the level of Ras protein expression (Fig. 1A) and the abundance of MMP-9 mRNA in the Ras transfectants. This observation was confirmed and extended with the MMP-9 enzyme activity assays described below.

v-Ha-Ras expression increased secreted 92-kDa gelatinolytic activity

To study the 92-kDa gelatinolytic activity, we performed zymographic analysis. This analysis separates proteolytic enzymes contained in conditioned media using a polyacrylamide gel under nonreducing conditions. Enzyme activities are identified by the presence of clear zones against a blue background. Because both the latent and active forms of MMP-9 are visible in this in-gel activity assay, the activity bands actually show the total activity of the MMP-9 enzyme. Figure 3A shows that Ras transfectants W13, U12, and U23 displayed dramatically increased secretion of MMP-9, but not MMP-2. These results demonstrated that U23 and U12 had characteristics of more invasive, malignant cells with respect to proteolytic enzyme activity. This was also consistent with other aspects of their malignant phenotype, such as shortened cell doubling time and higher plating efficiency [16].

We have demonstrated that Ras overexpression in REC cells can modify cellular ROS production, and that this is coupled with increased MMP-9 secretion. To study the relation between ROS level and MMP-9 secretion, we treated clone U23 with antioxidants including N-acetyl-cysteine (NAC) and glutathione monoester (GME), and then studied the level of MMP-9 secretion by U23 cells. Figure 3B shows that MMP-9 secretion from clone U23 was attenuated by the treatment of 5 mM N-acetyl cysteine (NAC) or 4 mM GME for 48 h. These results suggested that v-Ha-Ras overexpression induced MMP-9 expression in part through ROS alteration in REC cells.

v-Ha-Ras expression downregulated PAI-1 expression in REC cells

Plasminogen activator inhibitor-1 (PAI-1) regulates the activity of plasminogen activators by binding and forming enzymatically inactive complexes with them. Thus, PAI-1 indirectly regulates the production of plasmin. To determine the effects of v-Ha-Ras overexpression on PAI-1 gene expression, northern blot analysis was performed. The results, shown in Fig. 4A, revealed that v-Ha-Ras overexpression in U12 and U23 cell lines greatly decreased PAI-1 steady-state mRNA levels. This finding suggests that U12 and U23

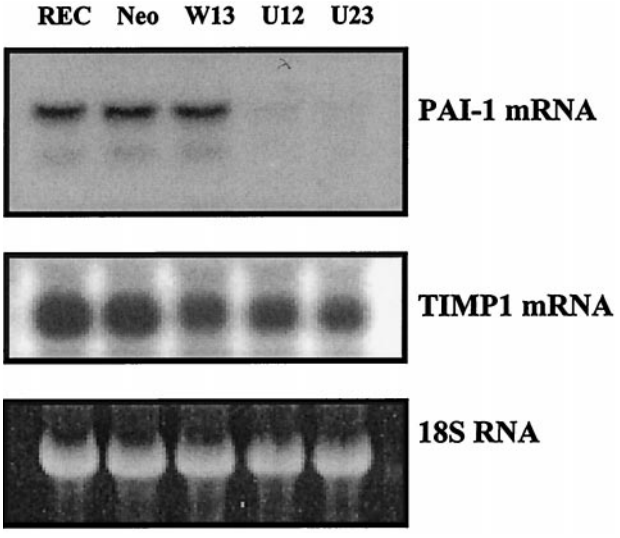

Fig. 4. Steady-state levels of both PAI-1 and TIMP-1 mRNA were downregulated in v-Ha-Ras overexpressing REC cells compared to parent or Neo control cells as determined by northern blotting. Eighteen μg of total RNA was fractionated on 1.5% agarose gel, transferred onto a nylon membrane, and hybridized with ^{32}P-labeled probes made from rat PAI-1 cDNA. After hybridization, the cDNA probe was stripped by boiling the membrane in 0.1%SDS for 2 $\times$ 15 min. Membrane was reprobed for ^{32}P-labeled rat TIMP-1 gene cDNA.

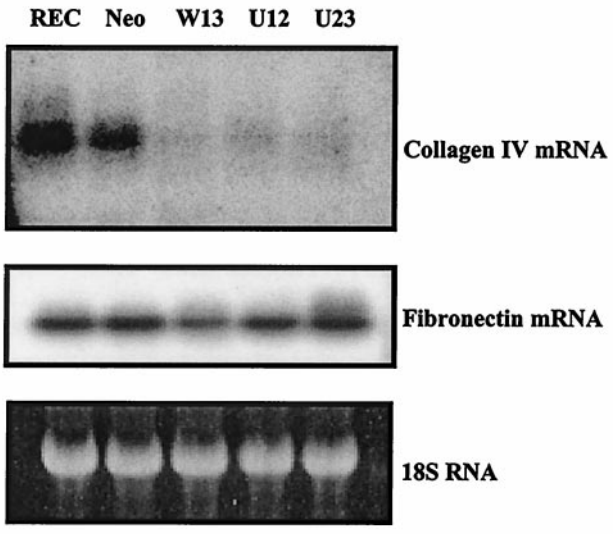

Fig. 5. Steady-state mRNA levels of collagen IV, but not fibronectin, were downregulated by v-H-Ras overexpression as determined by northern blotting. Eighteen μg of total RNA was fractionated on 1.5% agarose gel, transferred onto a nylon membrane, and hybridized with ^{32}P-labeled probe made from rat collagen IV gene cDNA. After hybridization and detection of the first signal, the cDNA probe was stripped by boiling the membrane in 0.1% SDS for 2 $\times$ 15 min. The membrane was reprobed with a ^{32}P-labeled cDNA probe specific for the rat fibronectin mRNA.

had lower PAI-1 mRNA levels, suggesting they would have higher plasminogen activators that could activate plasminogen into plasmin. Plasmin can further activate latent MMP-9 to activated MMP-9. Thus, low PAI-1 expression can indirectly increase MMP-9 activity, and this provides a plausible mechanism to explain the observed increase in MMP-9 gelatinolytic activity.

v-Ha-Ras transduction downregulated TIMP-1 expression in REC cells

MMP activities are inhibited by TIMPs that are expressed by a variety of cell types and are present in most tissues and body fluids. TIMP-1 is a glycoprotein with a molecular mass of 28.5 kDa. It binds to the active forms of MMP-9, as well as the pro-form of MMP-9 [22]. To determine the effects of v-Ha-Ras overexpression on TIMP-1 steady-state mRNA levels, northern blot analysis was performed. The results of that experiment, shown in Fig. 4B, revealed that v-Ha-Ras overexpression in U12 and U23 cell lines greatly decreased TIMP-1 gene expression. This result demonstrated that v-Ha-Ras overexpression not only increased MMP-9 secretion, but also decreased expression of the MMP-9 inhibitor TIMP-1, suggesting another mechanism for the observed increase in MMP-9 activity.

Steady-state mRNA levels of collagen IV, but not fibronectin, were downregulated by Ras overexpression

Collagen IV is the major component of basement membranes; malignant tumors consistently exhibit a defective base membrane where tumor cells invade the stroma [23]. To determine the effects of v-Ha-Ras overexpression on collagen IV steady-state mRNA levels, northern blot analysis was performed. Figure 5 demonstrates v-Ha-Ras overexpression downregulated collagen IV gene expression. Collagen IV mRNA levels were dramatically decreased in v-Ha-Ras-transduced cells W13, U12, and U23. In contrast, the steady-state mRNA level of fibronectin, another component of basement membranes, was not affected by v-Ha-Ras overexpression in these cells.

MMP-9 gene expression was in part regulated by cellular oxidant levels

Rat MMP-9 gene expression is regulated by transcription factors such as NF-κB and AP-1 [24]. The MMP-9 5′ regulatory region includes two AP-1 binding sites (-512 to -506, -87 to -81) and one NF-κB binding site (-569 to -562). We designed three oligonucleotides corresponding to these three regions and tested the DNA-binding activity of NF-κB and AP-1 in the Ras-transfected cells. The NF-κB gel mobility shift assay

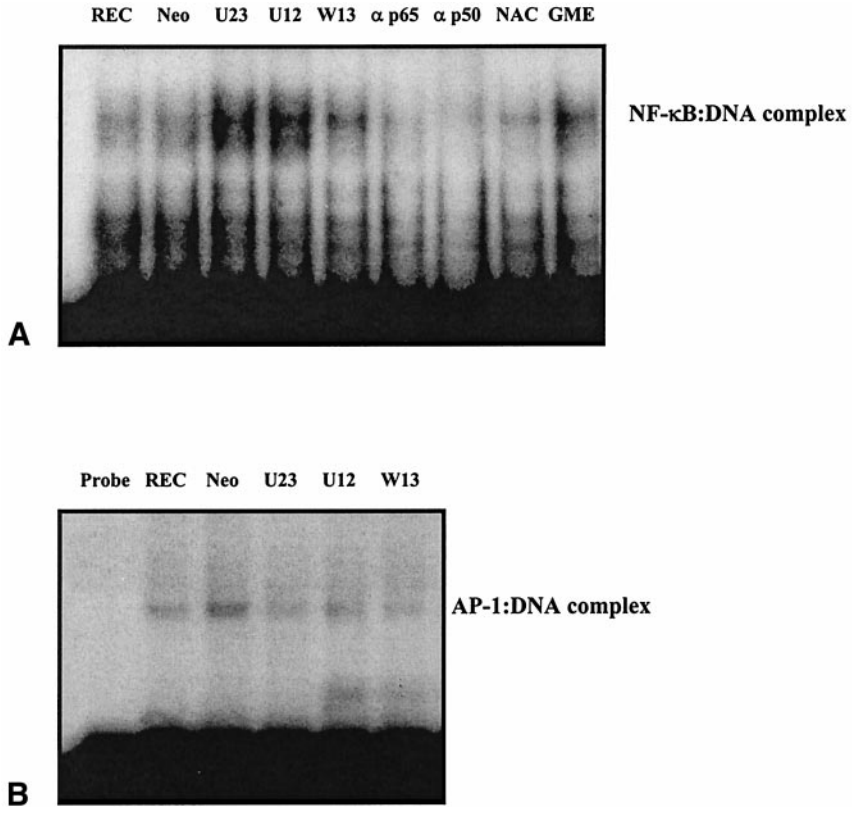

Fig. 6. NF-κB, but not AP-1, DNA-binding activities were enhanced by v-Ha-Ras overexpression and attenuated by antioxidant treatment. Gel mobility shift assay for NF-κB and AP-1 DNA-binding activity in MMP-9 gene regulatory region in Ras overexpressing REC. (A) NF-κB oligonucleotide is made from rat MMP-9 gene promoter −569 to −562 region; sense 5′-ACTGCCCGTGGAATTCCCCAA-3′ and anti-sense 5′-ACTGTTGGGGGAATTCCACGGG-3′. Antibodies against NF-κB subunit p50 or p65 (a p50 and a p65, respectively) were used for the supershift assays and both disrupted formation of the complex. U23 cells were further treated by 5 mM NAC, or 1 mM glutathione ester (GME), for 24 h before nuclear protein extraction. (B) AP-1 (1) DNA-binding activities were not enhanced by Ras overexpression in REC. AP-1 (1) oligonucleotide is made from rat MMP-9 gene promoter −512 to −506 region, sense 5′-ACTGGGAAGCTGAGTCAAAGAC-3′ and anti-sense 5′-ACTGGTCTTTGACTCAGCTTCC-3′. In both panels, 1.5 μg of nuclear protein for each sample was incubated with the ^{32}P-labeled oligonucleotide probe. The experiment was repeated twice and representative data are shown.

demonstrated that NF-κB DNA-binding activity was enhanced in Ras overexpressing clones W13, U12, and U23 (Fig. 6A), and that the degree of increased NF-κB binding correlated directly with Ras protein expression (Fig. 1A) and with MMP-9 activity (Fig. 3A). More importantly, when U23 cells were treated with antioxidants NAC (5 mM) or GME (4 mM) for 24 h, this NF-κB binding activity was inhibited (Fig. 6A). NAC can scavenge peroxides directly, and increase the cellular GSH pool, thereby decreasing the level of oxidants within the cell. GME is converted to glutathione in cells through cleavage of the ester group. GSH serves as a redox buffer for the cell and is the cofactor needed by glutathione peroxidase (GPx) to scavenge peroxides. The MMP-9 zymographic analysis (Fig. 3B) also showed that MMP-9 expression was partially inhibited by NAC and GME. These findings suggest that MMP-9 gene expression is regulated, at least in part, by cellular pro-oxidant levels. DNA-binding activities of AP-1 to both sites in the MMP-9 gene promoter region were weak. Figure 6B shows the result of AP-1 DNA-binding activity to one of these elements (−512 to −506). Similar results were obtained for DNA-binding activity to the other AP-1 site (−87 to −81) (data not shown). Thus, it appeared from these findings that AP-1 was less involved than NF-κB with the regulation of MMP-9 gene transcription in response to Ras-mediated production of cellular oxidants.

DISCUSSION

Reactive oxygen species (ROS) participate in the carcinogenic process at both the initiation and promotion steps of tumor development. Here, we demonstrated that v-Ha-Ras oncogene overexpression increased superoxide production in stably transfected cells and caused upregulated MMP-9 gene expression. Overexpression of v-Ha-Ras enhanced NF-κB, but not AP-1 DNA binding to motifs in the promoter region of MMP-9 gene. The

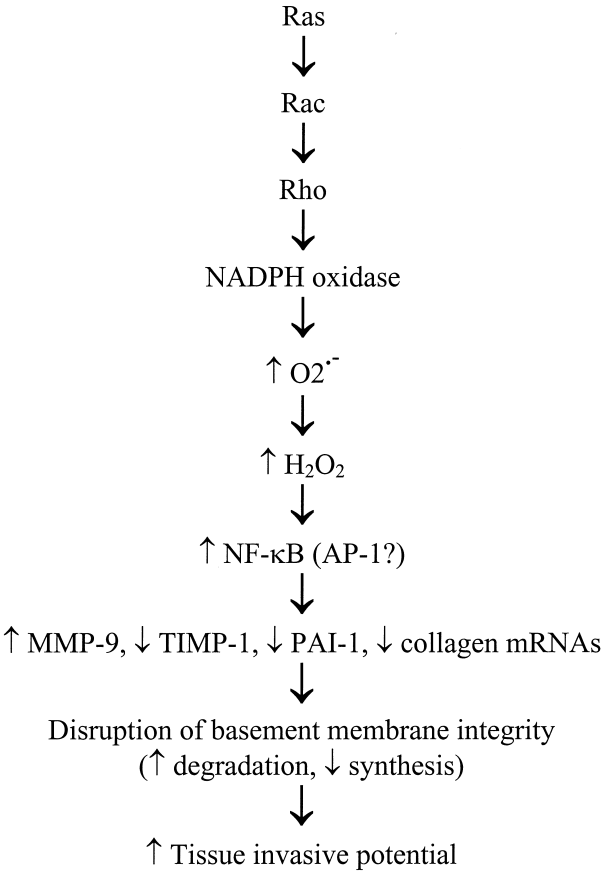

Fig. 7. Summary of a possible mechanism by which Ras overexpression or constitutive activation could lead to disruption of basement membrane integrity and increased invasive potential. Ras transduces signals downstream through a series of small GTP binding proteins, including Rac and Rho, that impinge upon a NADPH oxidase with the potential to produce superoxide. This serves as a major source of reactive oxygen involved in cell signaling. The superoxide is rapidly dismuted to hydrogen peroxide that can then serve as a second messenger-like signal to activate redox-sensitive transcription factors such as NF-κB and AP-1. These activated transcription factors lead to changes in gene expression that provide an overall gelatinolytic environment and lead to an imbalance in the degradation and synthesis of basement membrane components, culminating in increased tissue invasiveness.

NF-κB DNA binding could be attenuated by treatment with the antioxidants NAC and GME, which implied that intracellular oxidant levels could mediate MMP-9 expression. Taken together, our findings suggest that v-Ha-Ras overexpression in REC caused upregulated MMP-9 gene expression, at least in part, by increasing oxidant levels.

The basement membrane is a barrier composed of extracellular matrix that separates epithelia or endothelia from underlying connective tissue. Under normal conditions basement membranes are impermeable to large proteins and cells. However, basement membranes become locally permeable to cell movement during tissue remodeling and invasive processes [1]. Defective basement membrane organization or loss may be due to decreased synthesis, abnormal assembly, increased degradation of its components, or a combination of these mechanisms. In v-Ha-Ras overexpressing REC, we found that gene expression of collagen IV, a major component of basement membrane, was downregulated. At the same time, the expression and secretion of the basement membrane degrading metalloproteinase MMP-9 was upregulated. In addition, the expression of two key inhibitors of MMPs, PAI-1 and TIMP-1, was decreased. The net result is anticipated to enhance degradation of basement membranes and therefore is expected not only to facilitate tumor formation, but also tumor cell invasion to surrounding tissues.

ROS have been shown to play important roles in Ras mitogenic signaling. Ras overexpression causes superoxide production and alters the intracellular oxidant levels [19,20,25]. We also demonstrated that Ras-transduced REC generated large amounts of superoxide during this Ras associated malignant conversion. MMP-9 degrades basement membrane type IV collagen, and its expression is closely connected to cellular migration and invasion. Our gel mobility shift assay confirmed that MMP-9 gene promoter specific NF-κB DNA-binding activity was increased in v-Ha-Ras-transduced REC, which suggests that MMP-9 gene transcription could be upregulated by oxidant-induced NF-κB activity. Zymographic analyses demonstrated that MMP-9 was secreted at higher levels from v-Ha-Ras-transduced REC than parent cells or Neo controls. However, when Ras-transduced cells were treated with NAC, which can not only directly remove both superoxide and hydrogen peroxide, but also increase the intracellular GSH pool, MMP-9 gene promoter specific NF-κB DNA-binding activity was greatly inhibited. Glutathione monoester, which can increase the intracellular GSH pool after entering the cell and having the ester group removed, moderately inhibited this NF-κB DNA-binding activity. Taken together, these results suggest that MMP-9 gene expression is mediated at least in part by increasing oxidant levels, and further suggest that oxidant-induced NF-κB activity may be in part responsible for the increase in MMP-9 gene expression.

The idea that increased cellular oxidizing environments may be responsible for activation of gene expression and secretion of matrix-degrading metalloproteinases is beginning to become more widely appreciated. For example, it was recently shown that oxidative stress regulates matrix metalloproteinase activity as well as collagen expression in cardiac fibroblasts [26]. In addition, hydrogen peroxide-mediated activation of AP-1 has been suggested to play a role in the induction of matrix metalloproteinase-1in fibroblasts [27,28]. Similarly, sublethal exposure to H_2O_2 has been shown to increase the

expression and activation of MMP-2 in human endothelial cells [29]. Thus, in a variety of cell types, increases in the pro-oxidant to antioxidant balance appear to cause a shift from normal homeostasis and maintenance of ECM to one in which ECM is aberrantly degraded, as in the case of tissue remodeling and tumor cell invasion. These concepts are summarized in Fig. 7 in the context of the Ras signaling pathway.

Approximately 30% of all human tumors overexpress some kind of mutant Ras protein, which implies that superoxide production may be increased in these cells [30]. However, cancer cells in general express low constitutive levels of MnSOD and CuZnSOD when compared with their cells of origin [31], which suggests that cancer cells can maintain a relatively higher superoxide level. Under these conditions more hydrogen peroxide can be produced by dismutation of superoxide, and cellular oxidant levels can increase. The alteration of cellular oxidant levels can shift the balance of synthesis and degradation of basement membranes to yield a net loss of basement membrane ECM, thereby promoting tumor cell invasion and malignancy.

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ABBREVIATIONS

AP-1—activator protein-1
CuZnSOD—copper-and zinc-containing superoxide dismutase
ECM—extracellular matrix
GPx—glutathione peroxidase
JNK—c-jun N-terminal kinase
MAPK—Mitogen-activated protein kinase
MnSOD—manganese-containing superoxide dismutase
MMPs—matrix metalloproteinases
MMP-9—92-kDa type IV collagenase
$O_2^{\bullet -}$—superoxide anion radical
PAI-1—plasminogen activitor inhibitor-1
PMSF—phenylmethylsulfonyl fluoride
ROS—reactive oxygen species
SOD—superoxide dismutase
TIMP-1—tissue inhibitor of metalloproteinases-1