Oxidative Stress Regulates Vascular Endothelial Growth Factor-A Gene Transcription through Sp1- and Sp3-dependent Activation of Two Proximal GC-rich Promoter Elements*

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Enhanced VEGF-A (vascular endothelial growth factor A) gene expression is associated with increased tumor growth and metastatic spread of solid malignancies including gastric cancer. Oxidative stress has been linked to tumor-associated neoangiogenesis; underlying mechanisms, however, remained poorly understood. Therefore, we studied the effect of oxidative stress on VEGF-A gene expression in gastric cancer cells. Oxidative stress generated by HO2 application potently stimulated VEGF-A protein and mRNA levels as determined by enzyme-linked immunosorbent assay and real-time PCR techniques, respectively, and elevated the activity of a transfected (−2018) VEGF-A promoter reporter gene construct in a time- and dose-dependent manner (4–8-fold). These effects were abolished by the antioxidant N-acetylcysteine, demonstrating specificity of oxidative stress responses. Functional 5’ deletion analysis mapped the oxidative stress response element of the human VEGF-A promoter to the sequence −88/−50, and a single copy of this element was sufficient to confer basal promoter activity as well as oxidative stress responsiveness to a heterologous promoter system. Combination of EMSA studies, Sp1/Sp3 overexpression experiments in Drosophila SL-2 cells, and systematic promoter mutagenesis identified enhanced Sp1 and Sp3 binding to two GC-boxes at −73/−66 and −58/−52 as the core mechanism of oxidative stress-activated VEGF-A transactivation. Additionally, in Gal4-Sp1-/Sp3-Gal4-luciferase assays, oxidative stress increased Sp1 but not Sp3 transactivating capacity, indicating additional mechanism(s) of VEGF-A gene regulation. Signaling studies identified a cascade comprising Ras → Raf → MEK1 → ERK1/2 as the main pathway mediating oxidative stress-stimulated VEGF-A transcription. This study for the first time delineates the mechanisms underlying oxidative stress regulation of VEGF-A gene transcription by oxidative stress and thereby further elucidates potential pathways underlying redox control of neoangiogenesis.

Reactive oxygen species (ROS),1 such as superoxide O2−, hydroxyl radical OH·, and H2O2 are continuously generated as products of cellular metabolism (1–3). Increased levels of ROS reflecting oxidative stress have been observed during a number of physiologic and pathologic states, comprising cellular differentiation, reperfusion injury, and ulcerative and inflammatory conditions (2, 3). Oxidative stress is also believed to be a major pathogenic factor underlying development and progression of chronic disease states such as atherosclerosis, diabetes, and aging (2–4). Moreover, ROS participate in malignant transformation as well as metastatic spread of tumor cells (2–4). Elevated levels of ROS can lead to DNA damage and apoptosis but also act as important signaling molecules influencing various cellular functions including gene expression (2, 6–9).

The gastric mucosa is permanently exposed to luminal oxidants generated from ingested food, bacteria, and shed mucosal cells, and together with the surface mucus layer, the gastric epithelium represents the first line of defense against luminal oxidative alterations. Increased ROS levels have been linked to peptic lesions of the gastric mucosa triggered by ethanol, non-steroidal anti-inflammatory drugs, stress, ischemia-reperfusion, and Helicobacter pylori infection (10–12). In addition to these deleterious effects, ROS are also capable of influencing mucosal repair processes by stimulating epithelial proliferation, production, and release of mucosal growth factors as well as activation of proangiogenic pathways (13). Moreover, several studies have suggested that oxidative stress is also involved in gastric carcinogenesis (14, 15). Accordingly, epidemiological studies indicated that application of antioxidants can significantly reduce the risk of gastric cancer (15) and lead to regression of gastric epithelial metaplasia (16), a lesion believed to reflect premalignant states of the gastric mucosa.

Neoangiogenesis is a general pathophysiological mechanism critically involved in healing of inflammatory and ulcerative epithelial lesions as well as tumor growth and metastasis (17). Among the proangiogenic factors identified so far, VEGF-A (vascular endothelial growth factor A) represents one of the most potent stimuli of neoangiogenesis (17, 18). In the stomach, enhanced VEGF-A gene expression has been identified to critically contribute to peptic ulcer healing (13, 19–21). In addi-
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EXPERIMENTAL PROCEDURES

Cell Culture—AGS human gastric adenocarcinoma cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 2 mM glutamine (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin (Biochrom KG, Berlin, Germany), and 10% bovine calf serum (Biochrom KG) in a humidified atmosphere (5% CO2, 95% air). Cultures of Drosophila melanogaster Schneider cell line 2 (SL-2) were maintained in Schneider's Drosophila medium (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin at 25 °C and atmospheric CO2.

VEGF-A Enzyme-Linked Immunoprecipitation Assay Determinations in Cell Cultures—2 × 106 AGS cells were plated in 24-well plates in growth medium overnight and then switched to serum-free UltraCulture® medium (BioWhittaker Inc., Walkersville, MD) containing 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Conditioned medium was collected after incubation with or without 1 μM hydrogen peroxide (Sigma) for various times. To determine cellular VEGF-A production, cultured cells were treated with lysis buffer containing 2 mM EDTA, 20 mM Tris (pH 7.8), 150 mM NaCl, 50 mM β-glycerolphosphate, 0.5% Nonidet P-40, 1 mM dithiothreitol, 5 μg/ml aprotinin, 10 mM NaF, 2 μM leupeptin, and 2 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using the Protein Assay Kit® (Bio-Rad). VEGF-A concentrations were assessed using a commercial enzyme-linked immunoprecipitation assay (Quantikine™, R&D Systems, Minneapolis, MN), according to the manufacturer's instructions, and normalized to protein content. To exclude cell damage after H2O2 treatment, lactate dehydrogenase activity in cellular supernatants was measured using the CytoTox96® nonradioactive cytotoxicity assay kit (Promega, Mannheim, Germany).

TagMan® Quantitative Real-Time Reverse Transcription-PCR Analysis—Primers were designed using Primer Express software (PerkinElmer Life Sciences). Expression levels of human VEGF-A and housekeeping gene β-actin were determined using the following primer pairs: forward VEGF-A (5′-cttgctgtgctgtcaacc-3′) and reverse VEGF-A (5′-cacaagtcagctgttaag-3′); forward β-actin (5′-ctcaacccaggtatagcag-3′) and reverse β-actin (5′-cacaacagtctcttccg-3′). Specificity of the PCR products was demonstrated for each fragment by agarose gel analysis, gel electrophoresis, and sequencing. The SYBR Green I assay and the ABI Prism 7700 sequence detection system (Applied Biosystems) were used for detecting real-time quantitative PCR products from 0.25–2.5 ng of reverse-transcribed cDNA samples. SYBR Green I dye intercalation into the minor groove of double-stranded DNA reaches an emission maximum at 530 nm. PCRs for each sample were done in triplicate for both target gene and β-actin control. Quantitation of mRNA expression was carried out by relating the PCR threshold cycle obtained from tissue samples to a cDNA standard curve. In the case of the β-actin, the PCR threshold cycle was related to the number of AGS cells from which β-actin was extracted. The normalized amount of VEGF-A expression was obtained by dividing the average threshold cycle value by the averaged β-actin value of AGS cells and given in copies of VEGF-A per AGS cell.

Transfection Studies—Transient transfections of cultured AGS cells were carried out using the calcium phosphate precipitation technique (DNA transfection kit; 5 Prime → 3 Prime, Inc., Boulder, CO) as previously described (36–38). Briefly, AGS cells were transfected with 0.5 μg of reporter gene plasmid per well unless otherwise indicated. To correct for transfection efficiency, 50 ng/well of Renilla luciferase construct pRL-TK (Promega) were cotransfected. After transfection, cells were maintained in serum-free UltraCulture® (BioWhittaker) for 24 h. Unless otherwise indicated, cells were stimulated for 6 h with 500 μM hydrogen peroxide or 10 μM phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 2 h. Block p38 MAP kinase incubations were performed in the presence of 25 μM SB 202190 (Calbiochem). For transfection of SL-2 cells, 106 cells/well were transfected with 3 μg of VEGF-A (88–50)–Luc along with 1 μg of expression constructs (Sp1, Sp3, Sp1-DDD, Sp3-DDD) or empty vectors. Luciferase activities were detected in a monolight Luminometer (EG Berthold, Bad Wildbach, Germany) using the “Dual Luciferase Reporter Assay” (Promega). Incubations were performed in triplicates, and results were normalized for transfection efficiency and calculated as means ± S.E. Values were expressed as arbitrary light units or fold-increases in luciferase activity compared with controls. Statistical significances were calculated using Student’s t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

DNA Constructs and Reporter Plasmids—VEGF-A 5′-deletion luciferase constructs have previously been reported (33). To study VEGF-A regulatory elements in a heterologous promoter system, wild type and GC-box-mutated VEGF-A (88–50) oligonucleotides (Table I) were cloned at HindIII (5′) and XhoI (3′) restriction sites into construct pTS1-Luc (39). Constructs were confirmed by restriction analysis and dideoxysequencing. Reporter plasmid Gal4-Luc, in which the luciferase gene is driven by a multimer of the Gal4 yeast transcription factor-binding element (40), as well as transactivator constructs Gal4-Sp1 and Gal4-Sp3 have been described before (41). Expression constructs encoding wild type Sp1 or Sp3 or corresponding mutants lacking their transactivation domain (pPACSp1-DDD and pPACSp3-DDD) have also been described (42). Constructs encoding human ERK1, ERK2, MEK1, or Raf-1 have been used before (36, 38). Expression constructs encoding dominant-negative mutants of ERK1 (DN ERK1(R71F)), ERK2 (DN ERK2(R52R)), MKK4 (DN MKK4), Raf-1 (DN Raf-1), and Ras (DN Ras(N17)) have been previously employed (36, 38).

Electrophoretic Mobility Shift Assays—EMSA analysis of AGS nuclear extracts was performed as previously described (38). In brief, nuclear protein extracts (5 μg) were incubated with [γ-32P]ATP-radioabeled double-stranded oligonucleotides. DNA binding reactions were performed in a buffer containing 20 mM HEPES (pH 8.4), 1 μg of poly(dI-dC), 10 μg of bovine serum albumin, 60 mM KCl, 5 mM dithiothreitol, 100 μM znoCL, and 1 μM glicerol. For competition experiments, nuclear extracts were incubated with a 100-fold molar excess of double-stranded competitor oligonucleotides. For supershift experiments, nuclear extracts were incubated with 1 μl of anti-Sp1, anti-Sp3, anti-Sp4, anti-AP2, and anti-Egr-1 antibodies (Santa

ion, gastric adenocarcinomas were found to frequently display high levels of VEGF-A expression accompanied by increased intratumoral microvessel density (22, 23), whereas injection of VEGF-A-specific neutralizing antibodies were capable of potently inhibiting the growth of gastric cancer xenotransplanted in rodents (24). Despite the fact that these studies clearly established an important role of VEGF-A-dependent angiogenesis in mucosal regeneration, peptic ulcer healing, and gastric cancer, the pathways controlling VEGF-A gene expression in these settings have not yet been defined.

Previous studies revealed that VEGF-A gene expression can be influenced by extracellular growth factors, cytokines, and genetic alterations or hypoxia (17, 25). Moreover, current studies indicated that angiogenic processes may be influenced by cellular redox processes (26–30); potential mechanisms linking oxidative stress to proangiogenic factors like VEGF-A, however, remained unclear (29). Transcriptional activation of the VEGF-A promoter represents a core mechanism through which expression of the VEGF-A gene can be regulated (17, 18, 25). Several cis-acting promoter elements including a hypoxia-responsive site at −975, proximal GC-rich elements, and Egr-1 and AP2 recognition motifs were found to participate in VEGF-A gene regulation (31–35). Depending on the cellular context and/or the stimulus investigated, these recognition motifs and/or their respective binding factors variably contribute to VEGF-A expression control (31–35). Moreover, VEGF-A gene expression has been shown to be regulated through several signaling cascades comprising MAP kinase ERK-, JNK-, and p38-dependent cascades as well as NF-κB pathways (17, 18, 25, 32). In contrast, signaling pathways mediating the effects of oxidative stress on the VEGF-A gene await clarification.

Here we demonstrate that oxidative stress generated by the model oxidant H2O2 at submillimolar concentrations potently stimulates release and production of VEGF-A in gastric cancer cells and provides evidence that transcriptional activation of the VEGF-A promoter represents the underlying core mechanism. Moreover, we identify the zinc finger transcription factors Sp1 and Sp3 as molecular mediators of the VEGF-A redox response and show that Sp1/Sp3-activated GC-boxes located in the proximal VEGF-A promoter differentially participate in oxidative stress-dependent VEGF-A gene regulation. Furthermore, we show that MAPK ERK-related and to a lesser extent JNK-related signaling pathways are crucial for transmission of oxidative stress effects on the human VEGF-A gene in gastric cancer cells.
Cruz Biotechnology, Inc., Santa Cruz, CA). DNA-protein complexes were electrophoresed in 6% non-denaturing polyacrylamide gels containing 0.5% TBE (50 mM Tris, 50 mM borate, 2 mM EDTA). Gels were dried and exposed to Eastman Kodak Co. BioMax MR films (Amersham Biosciences) using an intensifying screen.

**Immunoblotting**—For signaling studies, AGS cells were exposed to 1 mM H₂O₂ and whole cell lysates were prepared in 20 mM Tris (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 10 mM K₂HPO₄, 1 mM Na₃VO₄, 10 mM NaF, 1.25% Nonidet P-40, and 10% glycerol as described (38). Following SDS-PAGE and transfer to nylon membranes, proteins were electrophoresed in 6% nondenaturing polyacrylamide gels containing 0.5 mM H₂O₂, and whole cell lysates were prepared in 20 mM Tris (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 10 mM K₂HPO₄, 1 mM Na₃VO₄, 10 mM NaF, 1.25% Nonidet P-40, and 10% glycerol as described (38). Gels were dried and exposed to Eastman Kodak Co. BioMax MR films (Amersham Biosciences) using an intensifying screen.

**RESULTS**

**Oxidative Stress Stimulates Production and Release of VEGF-A Protein and Increases VEGF-A mRNA Levels in AGS Cells**—To determine the effects of oxidative stress on VEGF-A gene expression, AGS cells were exposed to H₂O₂ and analyzed for VEGF-A protein and mRNA levels. H₂O₂ time- and dose-dependently stimulated production and release of VEGF-A protein, having a maximum (3-fold elevation) at 12 h (Fig. 1, A and B). These responses were redox-specific, as shown by their complete reversal through application of the antioxidant N-acetylcysteine. To investigate potential cell lysis due to H₂O₂ treatment, lactate dehydrogenase activity in cell culture supernatants was analyzed in parallel (Fig. 1C) and found to be unaltered (Fig. 1C). Similar to the changes observed on the protein level, H₂O₂ treatment increased VEGF-A mRNA levels (maximum at 8–12 h), suggesting that enhanced transcription represents the mechanism underlying oxidative stress responsiveness of the VEGF-A gene (Fig. 1D).

**Oxidative Stress Potently Transactivates the VEGF-A Gene Promoter**—To determine transactivating effects of oxidative stress on the VEGF-A gene, functional transfection studies using VEGF-A luciferase reporter gene constructs were performed. H₂O₂ treatment of AGS cells potently stimulated the VEGF-A promoter in a dose-dependent manner, with maximal stimulation observed at 750 μM H₂O₂ (Fig. 2A). Analysis of time-response relationships demonstrated maximal VEGF-A transactivation after 4–8 h of H₂O₂ exposure (Fig. 2B), which corresponds well with the results obtained in VEGF-A mRNA studies (Fig. 1D).

**Oxidative Stress Regulates the VEGF-A Promoter through a Proximal 39-bp Promoter Element**—To identify promoter regions mediating oxidative stress responsiveness of the human VEGF-A gene, functional 5’ deletion analysis was performed. These studies revealed that loss of the region spanning −85 to −8 had no substantial influence on VEGF-A promoter activity. In contrast, removal of an additional 33 nucleotides abolished basal and H₂O₂-stimulated VEGF-A promoter activity (Fig. 3A), suggesting that −85/−52 comprises essential regulatory elements. To investigate the enhancer properties of this promoter region in detail, it was transferred to a heterologous promoter system (pT81-Luc) and functionally analyzed (Fig. 3B). The presence of the −88/−50 element conferred elevation of basal transcriptional activity (10–15-fold) as well as H₂O₂ responsiveness to the per se redox-insensitive pT81-Luc vector (Fig. 3B). Redox responses of VEGF-A −88/−50 were abolished by the antioxidant N-acetylcysteine (Fig. 3B). Analysis of the −88/−50 promoter region in EMSA studies showed that under basal and H₂O₂-stimulated conditions, two major
complexes were bound to this element (Fig. 3C) and that H₂O₂ treatment of AGS cells resulted in a parallel increase of complex I and II (Fig. 3C, lanes 3 and 4). Only infrequently we observed a third complex (complex III), which was not influenced by any oligonucleotides used in EMSA studies (data not shown) and therefore must be regarded as nonspecific. H₂O₂ treatment maximally enhanced complex I and II after 10 min, whereas prolonged exposure decreased complex formation (lanes 5–8). Under identical conditions, H₂O₂ did not increase the binding of CREB, USF-1, and USF-2 transcription factors to a minimal cox-2 (cyclooxygenase 2) promoter fragment, demonstrating that oxidative stress-triggered protein/DNA interaction at the VEGF-A promoter is not a random nonspecific process. 

Oxidative Stress Stimulates Binding of Sp1 and Sp3 to the VEGF-A Promoter—To further characterize the nuclear factors binding to the VEGF-A(−88/−50) element, EMSA competition studies were carried out (Fig. 4). The selection of consensus oligonucleotides used in these studies (Sp1, AP2, and Egr-1) was based on the presence of these sequences within the −88/−50 sequence. Under all conditions investigated, responses to H₂O₂ were statistically significant.

AP2 consensus sequence was clearly less effective (lanes 6 and 13), whereas Egr-1 consensus oligonucleotides had no effect (lanes 4 and 11). To confirm these results, EMSA supershift experiments using specific antibodies recognizing Sp1, Sp3, Sp4, AP2, or Egr-1 were performed. These studies identified complex I as containing Sp1 protein (Fig. 4, B lanes 3 and 8) and C (lanes 2, 5, 6, and 81), whereas complex II was shown to consist of Sp3 (Fig. 4C, lanes 3, 5, 7, and 8). In contrast, zinc finger protein Sp4 as well as AP2 and Egr-1 were not detected. In control EMSAs, the specificity of the AP2 antibody was confirmed using HeLa cell nuclear extracts together with a labeled AP2 consensus site as a probe (data not shown). Functionality of the Egr-1 antibody was confirmed in supershifts employing AGS nuclear extracts together with a proximal fragment of the mouse chromogranin A gene promoter as radiolabeled probe (38).

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2 M. Höcker, manuscript in preparation.
Fig. 4. Oxidative stress stimulates binding of Sp1 and Sp3 to the VEGF-A promoter. A, nuclear extracts of untreated (lanes 1–7) and stimulated AGS cells (500 μM H₂O₂ for 10 min) (lanes 8–14) were incubated with the probe VEGF-A(−88/−50) in the absence or presence of unlabeled double-stranded oligonucleotides representing VEGF-A(−88/−50) as well as Sp1, Egr-1, or AP2 consensus sequences. Application of mutant oligonucleotides served as a control. B and C, specific antibodies recognizing Sp1, Sp3, Egr-1, or AP2 were incubated with unstimulated (lanes 1–5) or H₂O₂-stimulated nuclear extracts (lanes 6–10) and radiolabeled VEGF-A(−88/−50) probe. The arrows indicate specific complexes. Data shown represent a typical result obtained from a series of three independent experiments.

Sp1 and Sp3 Potently Transactivate the VEGF-A(−88/−50) Element—After identification of Sp1 and Sp3 binding to the VEGF-A(−88/−50) element, we next investigated the functional impact of both transcription factors on this element in transient transfections. For this purpose, we employed Drosophila SL-2 cells, a cell model lacking endogenous Sp factors and therefore allowing investigation of gene regulation without interference of endogenous Sp proteins (41). Overexpression of either Sp1 or Sp3 potently stimulated the VEGF-A(−88/−50) element (7–8-fold), demonstrating that the VEGF-A promoter is highly reactive to both transcription factors (Fig. 5). Cotransfection of both factors did not yield in higher effects compared with the expression of the individual factors. Sp1 and Sp3 mutants lacking their DNA-binding domain exhibited virtually no transactivating effect on the VEGF-A(−88/−50) element (Fig. 5), confirming the specificity of results obtained with wild type expression constructs.

Oxidative Stress Stimulates Sp1 but Not Sp3 Transactivating Capacity—To investigate the influence of oxidative stress on the transactivating capacity of Sp1 and Sp3, we cotransfected AGS cells with Gal4-Sp1 or Gal4-Sp3 expression constructs and the 5′×Gal4-Luc reporter plasmid (Fig. 6). Whereas transfection of 5′×Gal4-Luc or Sp1/Sp3 transactivator plasmids alone produced reporter gene activity close to background levels, cotransfection of either Gal4-Sp1 or Gal4-Sp3 along with the 5′×Gal4-Luc reporter plasmid significantly increased basal promoter activity (−50-fold). Moreover, oxidative stress clearly increased Sp1 transactivating capacity (~3-fold) but did not significantly influence Sp3. These data confirm the regulatory influence of oxidative stress on Sp1 and suggest potential differences in the regulatory mechanisms controlling the activity of Sp1 and Sp3 in response to cellular oxidative stress in gastric cancer cells.

Proximal GC-boxes Differentially Contribute to VEGF-A Promoter Regulation—To elucidate the functional importance of individual GC-boxes located within the −88/−50 region, a series of VEGF-A(−88/−50) mutants was characterized in transfection assays and EMSA studies (Fig. 7). Compared with the wild type VEGF-A(−88/−50) element, mutation of GC-box I (Mut 1) did not affect oxidative stress-triggered activation of this sequence (Fig. 7A). Functional loss of GC-box II or GC-box III, however, significantly inhibited basal and stimulated promoter activity (−50-fold). Moreover, oxidative stress clearly increased Sp1 transactivating capacity (~3-fold) but did not significantly influence Sp3. These data confirm the regulatory influence of oxidative stress on Sp1 and suggest potential differences in the regulatory mechanisms controlling the activity of Sp1 and Sp3 in response to cellular oxidative stress in gastric cancer cells.

FIG. 5. Sp1 and Sp3 potently transactivate the VEGF-A(−88/−50) element. Drosophila SL-2 cells were transiently transfected with VEGF-A(−88/−50)-Luc or empty vector pT81-Luc along with expression constructs encoding Sp1 and/or Sp3. Cells were harvested after 24 h and assayed for luciferase activity. Results are expressed as -fold increase of control and represent mean ± S.E. of three separate experiments. The asterisks indicate statistically significant differences. **, p < 0.01.
respectively. Cells were treated with H2O2 or PMA at the indicated concentrations or left untreated. Results are expressed as arbitrary light units (A.U.) and represent mean ± S.E. of three separate experiments. The asterisks indicate statistically significant differences. *, p < 0.05; **, p < 0.01.

with either GC-box II (Mut 2) or GC-box III (Mut 7), promoter activity was decreased to a similar degree as observed after individual mutagenesis of GC-boxes II or III (~50%), further confirming the lack of functional relevance of GC-box I in VEGF-A (~88–50) regulation. EMSA studies using mutated VEGF-A (~88–50) oligonucleotides either as competitors (Fig. 7B) or as radiolabeled probes (Fig. 7C, data only shown for mutants Mut 1–3) revealed that mutation of individual GC-boxes I, II, or III had little or no effect on factor binding, supporting the concept that the loss of single GC-boxes can be compensated regarding Sp1/Sp3 binding by those GC-boxes remaining intact (Fig. 7, B (lanes 2, 5, and 7) and C (lane 2)). Examination of double mutations VEGF-A (~88–50) Mut 2, Mut 5, and Mut 7 showed reduction of Sp factor binding to variable degrees (Fig. 7, B and C, Mut 2), whereas alteration of all three GC-boxes abolished the ability of VEGF-A (~88–50) to bind nuclear proteins (Fig. 7, B and C, Mut 3). Interestingly, mutation of GC-box I together with either GC-box II (Mut 2) or GC-box III (Mut 7) showed only little effect on factor binding (Fig. 7B and data not shown), which correlates with the retained function of these mutants in transfection assays (Fig. 7A). In contrast, double mutation of GC-boxes II and III clearly reduced Sp1/Sp3 binding (Fig. 7, B (lane 5) and C (lane 3)), being in accordance with the reduced functional activity of this mutant. Together, these data strongly suggest that GC-boxes II and III are indispensable for basal and oxidative stress-triggered activity of the VEGF-A (~88–50) element, whereas GC-box I, which also possesses the capacity to bind Sp1/Sp3 transcription factors, has obviously no functional importance in this context.

Analysis of Signaling Cascades Mediating Oxidative Stress Responsiveness of the VEGF-A Promoter—To explore the signaling pathways activated by oxidative stress, the influence of H2O2 on the phosphorylation status of key kinases related to redox-triggered signaling was investigated. Exposure of AGS cells to oxidative stress led to a rapid increase in ERK1/2, JNK, and p38 kinases phosphorylated (Fig. 8A), reaching maximal effects after 5–10 min and sustained hyperphosphorylation throughout the entire experimental period of 120 min. Similarly, the upstream ERK1/2 kinase MEK1 was hyperphosphorylated in response to oxidative stress, showing a similar time-response relationship. After identification of MEK1/2/ERK1/2, JNK, and p38 as signaling targets of oxidative stress, we examined the functional role of these kinases for VEGF-A promoter regulation in transfection assays employing DN kinase mutants (Fig. 8B). Application of DN ERK1 and/or DN ERK2 decreased H2O2 responsiveness of the VEGF-A promoter by 40–50% (Fig. 8B), whereas the PMA response was almost abolished. Similarly, functional impairment of Raf-1 or Ras, both of which have been located upstream of the MEK1/2 → ERK1/2 signaling module, potently inhibited H2O2- and PMA-dependent VEGF-A promoter activation (~60–70% reduction). In con-

![Fig. 6. Oxidative stress stimulates Sp1 but not Sp3 transactivating capacity. AGS cells were transiently transfected with Gal4-Luc alone or Gal4-Sp1 or Gal4-Sp3 constructs, respectively. Cells were treated with H2O2 or PMA at the indicated concentrations or left untreated. Results are expressed as arbitrary light units (A.U.) and represent mean ± S.E. of three separate experiments. The asterisks indicate statistically significant differences. *, p < 0.05; **, p < 0.01.](http://www.jbc.org/)

![Fig. 7. Proximal GC-boxes differentially contribute to VEGF-A promoter regulation. GC-box mutations were introduced into the promoter fragment ~88–50, subcloned into the enhancerless reporter vector pT81-Luc, and used in transfections. AGS cells were stimulated with H2O2 (500 μM) or left untreated. Data are expressed as arbitrary light units, and -fold increases in response to H2O2 are additionally given on the right. Results represent means ± S.E. of three separate experiments, and asterisks indicate statistically significant differences. *, p < 0.05; B, EMSA competition studies were performed with AGS cell nuclear extracts and VEGF-A (~88–50) as radiolabeled probe. Mutant VEGF-A (~88–50) oligonucleotides were used as cold competitors as indicated. Data shown represent a typical result obtained from a series of three independent experiments. C, crude nuclear protein extracts of AGS cells were incubated with double-stranded, radiolabeled DNA probes representing VEGF-A (~88–50) mutants Mut 1, Mut 2, and Mut 3 (lanes 2–4) or wild type sequence (lane 1). Supershift studies employed anti-Sp1 or anti-Sp3 antibodies as indicated. Data shown represent a typical result obtained from a series of three independent experiments.](http://www.jbc.org/)
tric cancer cells and provide clear evidence that oxidative stress regulates VEGF-A gene expression through transcriptional mechanisms. Previous studies in epithelial and nonepithelial cell models suggested a link between the cellular redox status and enhanced VEGF-A production and/or secretion (28–30); underlying molecular determinants, however, have not yet been clarified. To provide a detailed functional analysis of molecular pathways mediating the effects of oxidative stress on the human VEGF-A gene, we initially investigated participating cis- and trans-activating factors employing 5′ deletion analysis of the VEGF-A promoter, DNA element transfer studies, and systematic core promoter mutagenesis. These studies revealed that a region spanning −88 to −50 is indispensable for basal as well as oxidative stress-triggered VEGF-A promoter activity. This GC-rich promoter region, which comprises consensus binding elements for Sp-like zinc finger proteins as well as AP2 and Ergr-1 transcription factors, has been demonstrated to be involved in VEGF-A gene regulation in various cell systems including fibroblasts (32, 33), keratinocytes (34), and glioma cells (35). Our study revealed that in gastric cells the −88/−50 element is bound by the zinc finger transcription factors Sp1 and Sp3 (Fig. 4). Application of oxidative stress increased DNA-protein complex formation at the −88/−50 site, strongly suggesting that enhanced binding of Sp1/Sp3 to the VEGF-A promoter represents an important mechanism through which oxidative stress transactivates the VEGF-A gene (Fig. 3). This mechanism is clearly different from previous observations made in platelet-derived growth factor-stimulated human fibroblasts and tumor growth factor-α-stimulated skin keratinocytes, showing constitutive binding of Sp1 and/or Sp3 to the proximal VEGF-A promoter without detectable changes upon stimulation (33, 34). Enhanced binding of Sp1 to the proximal VEGF-A promoter has been observed after activation of the MAPK/ERK signaling pathway in hamster fibroblasts (32). In this cell type, however, Sp1 was found to functionally cooperate with the transcription factor AP2, whereas Sp3 was not involved (32). Therefore, oxidative stress-triggered transactivation of the VEGF-A promoter through enhanced binding of both Sp1 and Sp3 represents a novel molecular mode of VEGF-A transactivation.

Transcription factor Sp1 belongs to the superfamily of Sp-like zinc finger proteins and has been implicated in the regulation of constitutively expressed “housekeeping genes” as well as genes influencing growth and differentiation (for a review, see Ref. 43). In addition, current studies demonstrated that Sp1 also participates in the regulation of inducible gene expression and that interaction of Sp1 with other transcription factors and/or cofactors such as CREB-binding protein, p300, or CRSP84 may represent an important transcriptional control mechanism (43). More recently, it became clear that Sp1 can also be regulated through changes of its phosphorylation state (44), and subsequently, different signaling pathways including Ras-dependent activation of the MEKK1/ERK cascade have been identified to target Sp1 (45–47). Similar to Sp1, Sp3 represents a zinc finger transcription factor comprising highly conserved DNA-binding domains, but analysis of their functional properties revealed significant differences between these two proteins (42, 43). Sp3 activates GC-rich DNA elements with affinities similar to other Sp proteins (42, 43). In some systems, however, Sp3 has been described to lack intrinsic activity and accordingly can act as transcriptional repressor of other transcription factors binding to the same element (42, 43). As demonstrated by experiments in SL-2 Schneider cells, which represent the appropriate cell model for analysis of Sp factor-dependent effects, Sp1 and Sp3 exert equiportent transactivating properties on the VEGF-A promoter, without any indication of Sp3 acting...
as an inhibitor of Sp1-dependent effects (Fig. 5). To explore the influence of oxidative stress on the transactivation capacity of Sp1 and Sp3, we employed appropriate Gal4-Sp1- or Gal4-Sp3/Gal4-luciferase cotransfection systems (48, 49). Interestingly, we observed that application of oxidative stress enhanced the transactivating capacity of Sp1 but not of Sp3, suggesting potential differences in the regulatory mechanisms controlling the activity of these transcription factors in response to cellular oxidative stress (Fig. 6). Recent studies demonstrated that the transactivating capacity of Sp3 can be suppressed through SUMO-dependent modification of the transcription factor (49, 50). To what extent this mechanism also contributes to regulation of Sp3 in context of VEGF-A gene regulation remains to be explored in future studies.

Previous studies demonstrated that depending on the cellular context and/or the stimulus investigated, the VEGF-A promoter can also be bound and transactivated by non-Sp transcription factors (30, 34). In contrast to these studies, in gastric cancer cells, no transcription factors other than Sp1 and Sp3 were detected to bind to the VEGF-A−88/−50 region. Initial competition studies using unlabeled oligonucleotides representing consensus binding sites for candidate transcription factors binding to the −88/−50 region suggested that AP2 may be interacting with this element (Fig. 4A, lane 13). Detailed analysis of this phenomenon, however, revealed that in AGS cells the AP2 consensus sequence binds Sp transcription factors (data not shown), and therefore, its effect in EMSA competition studies reflects interaction with Sp1 and Sp3 binding.

After identifying the proximal GC-rich site at −88/−50 as the critical promoter element mediating VEGF-A oxidative stress responsiveness, we aimed to define the functional properties of three GC-boxes as putative Sp1/Sp3 binding sites located within the −88/−50 region. For this purpose, a systematic mutational analysis of these elements was performed in functional transfection studies. In parallel, the influence of these mutations on transcription factor binding was investigated in EMSAs. We found that the structural integrity of the two most 3′-located GC-boxes II and III is indispensable for full basal and oxidative stress-stimulated VEGF-A promoter activity, whereas the 5′-located GC-box I has only minor importance for VEGF-A gene regulation in gastric cancer cells (Figs. 7 and 9). To our knowledge, this mapping of structure/function relationships within the human VEGF-A core promoter employing a combination of EMSA techniques and functional promoter studies represents the first detailed evaluation of Sp/KLF binding sites for basal and stimulated transcriptional regulation of the human VEGF-A gene. A similar approach had currently been used to characterize the importance of the proximal Sp factor binding sites for basal VEGF-A gene regulation in pancreatic cancer cells (31). This study, however, focused entirely on stimulus-independent VEGF-A transcription and GC-box mutagenesis, was less complete, not clearly allowing the determination of how individual promoter elements contribute to VEGF-A transcription (31).

Epithelial oxidative stress responses comprise activation of various signaling cascades including the proliferation-associated MAPK-ERK pathway as well as stress-related MAPK-JNK and p38 kinase cascades (2, 5–7). In gastric cancer cells, Ras-dependent activation of the Raf → MEK1 → ERK1/2 kinase module has been shown to be a major pathway through which oxidative stress can influence transcriptional responses (37). Our current study shows that H2O2 treatment of gastric cancer cells at a concentration of 1 mM led to increased phosphorylation of ERK1/2, MEK1, p38, and JNK, indicating activation of associated upstream kinases (Fig. 8A). The finding that JNK is hyperphosphorylated in response to H2O2 differs from our previous observations (37) and may be explained by the fact that H2O2 concentrations required for VEGF-A transcription were at least 2.5-fold lower than the doses at which stimulation of HDC promoter activity was obtained (2.5–10 mM). In full agreement with our previous work, functional studies revealed that the Raf-1/MEK1/ERK1/2 kinase module represents the major pathway through which oxidative stress transactivates the VEGF-A promoter. This view is supported by the finding that interruption of this cascade at different levels resulted in substantial impairment of H2O2-triggered VEGF-A promoter activity (70–80% inhibition; Fig. 8B).

Conversely, activation of the signaling cascade upstream of ERKs by overexpression of Raf or MEK, respectively, resulted in robust transcriptional VEGF-A responses, which were also obtained after ERK1 and/or ERK2 overexpression (Fig. 8C). Additionally, application of an inhibitory Ras mutant (N15) substantially impaired the effect of H2O2 on the VEGF-A promoter, confirming our previous observation that oxidative stress activates the Raf-1/MEK1/ERK1/2 kinase cascade in gastric cancer cells through (a) Ras-dependent mechanism(s) (37). These findings are compatible with the concept of the Raf/MEK1/ERK1/2 cascade being a critical signaling in VEGF-A gene regulation as shown in other experimental systems including acidic pH challenge of human glioma cells (51), insulin-like growth factor-I stimulation of NIH3T3 fibroblasts (52), and hamster fibroblast models (30). The finding that H2O2 stimulated JNK phosphorylation, whereas functional interruption of JNK signaling by application of a dominant negative MKK4 mutant reduced VEGF-A promoter activity by ≤30% (Fig. 8B), is well compatible with the MKK4/JNK module acting as additional signaling route mediating VEGF-A oxidant responsiveness in gastric cells (Fig. 9). Similar to these findings, evidence for dual regulation of VEGF-A expression through both MAPK-ERK and JNK pathways has been observed in primary rat astrocytes.
and glioblastoma cells exposed to ionizing radiation (53) as well as up-regulation of the GADD45 gene in response to UV radiation (54).

Together, identifying oxidative stress as a potent stimulus of VEGF-A gene expression in gastric cancer cells, our results support the concept that changes in the cellular redox status can directly exert regulatory effects on proangiogenic processes. Furthermore, the detailed delineation of pathways and structural elements mediating oxidative stress responsiveness of the VEGF-A gene can help to develop novel therapeutic approaches to target VEGF-A gene expression in benign and/or malignant (patho)biological settings.

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