N-myc Downstream-regulated Gene 1 Promotes Tumor Inflammatory Angiogenesis through JNK Activation and Autocrine Loop of Interleukin-1α by Human Gastric Cancer Cells*\(^\text{1,3}\)

**Background:** Expression of N-myc downstream-regulated gene 1 (NDRG1) is significantly correlated with tumor angiogenesis by human gastric cancer. NDRG1 overexpression induced JNK activation and expression of IL-1α and angiogenic CXC chemokines accompanied by tumor angiogenesis. NDRG1 promoted IL-1α-induced tumor angiogenesis through JNK/AP-1 activation. NDRG1/JNK/IL-1 axis could be a useful target for development of a novel anti-angiogenic strategy in gastric cancer.

The expression of N-myc downstream-regulated gene 1 (NDRG1) was significantly correlated with tumor angiogenesis and malignant progression together with poor prognosis in gastric cancer. However, the underlying mechanism for the role of NDRG1 in the malignant progression of gastric cancer remains unknown. Here we examined whether and how NDRG1 could modulate tumor angiogenesis by human gastric cancer cells. We established NU/Cap12 and NU/Cap32 cells overexpressing NDRG1 in NUGC-3 cells, which show lower tumor angiogenesis in relationship to NDRG1-induced inflammatory stimuli by gastric cancer cells.

N-myc downstream-regulated gene 1 (NDRG1)\(^*\) encodes a 43-kDa protein known as calcium-associated protein 43 (Cap43) or as reducing agents and tunicamycin-responsive protein (RTP/rit42) or as differentiation-related protein-1 (Drg-1) (1, 2). NDRG1 is phosphorylated and activated by serum- and glucocorticoid-regulated kinase 1 (SGK1) at its Ser/Thr, and is further phosphorylated by glycogen synthase kinase 3 (GSK-3), suggesting that dynamic changes occur in mammalian cells (2, 3). NDRG1 is also categorized as a metastasis-suppressor (4), but this function remains unclear. Expression of NDRG1 is higher in well differentiated cancer cells than in poorly differentiated cancer cells in colon, prostate, breast, and pancreatic cancer, suggesting a close association of NDRG1 with differentiation status in these human malignancies (5–10). NDRG1 expression is predictive of good prognosis in patients with cancers of the breast, colon, prostate, pancreas, and neuroblastoma (1, 2, 6, 9, 11–13). However, it is predictive of poor prognosis in patients with cancers of the liver, esopagus, and cervix (14–16). Its use as a predictive marker of either good or poor

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**Results:** NDRG1 overexpression induced JNK activation and expression of IL-1α and angiogenic CXC chemokines accompanied by tumor angiogenesis.

**Conclusion:** NDRG1 promoted IL-1α-induced tumor angiogenesis through JNK/AP-1 activation.

**Significance:** NDRG1/JNK/IL-1 axis could be a useful target for development of a novel anti-angiogenic strategy in gastric cancer.

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\(^1\) This article contains supplemental Table S1.

\(^2\) The abbreviations used are: NDRG1, N-myc downstream-regulated gene 1; GSK-3, glycogen synthase kinase-3; MMP, matrix metalloproteinase; ATF-3, activating transcription factor 3; AP-1, activator protein 1.
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prognosis in cancer patients depends upon the tumor species, histological type, and differentiation status of human malignancies.

NDRG1 is well known as a metastasis suppressor in both prostate and colorectal cancer (1, 2). In prostate cancer cells, NDRG1 induced expression of a metastasis suppressor gene named KAI1 through NDRG1-dependent attenuation of activating transcription factor 3 (ATF3)-nuclear factor-κB (NF-κB) pathway (17). Furthermore, NDRG1 suppressed metastasis by prostate cancer cell through a Wnt/β-catenin signaling pathway (18). A relevant study also demonstrated that NDRG1 blocked TGFβ-induced epithelial mesenchymal transition through modulation of Wnt/β-catenin signaling in colorectal cancer (19). We previously reported that NDRG1 was predictive of good prognosis in pancreatic cancer patients (9). Overexpression of NDRG1 resulted in suppression of tumor growth and angiogenesis, and inhibited the expression of the potent angiogenic factors IL-8/CXCL8, VEGF-A, and matrix metalloproteinase 9 (MMP-9) by pancreatic cancer cells (9). We also showed that NDRG1 overexpression resulted in reduced expression of CXC chemokines that promote recruitment of neutrophils and macrophages, and that attenuation of the NF-κB-IκBα signaling pathway was partly involved in the NDRG1-induced suppression of tumor angiogenesis and recruitment of tumor-associated macrophages to tumors (20, 21).

On the other hand, concerning the clinical significance of NDRG1 in human gastric cancer, Imagaki et al. (22) reported a significant correlation between NDRG1 expression and poor prognosis in gastric cancer patients. Consistent with this study, we have reported that higher expression of NDRG1 was predictive for poor prognosis and tumor angiogenesis in gastric cancer patients (23). We have further demonstrated that NDRG1 promoted peritoneal dissemination and ascites formation by aggressive gastric cancer cells through Snail-dependent epithelial mesenchymal transition (24). Together with those findings, it seems likely that NDRG1 promotes tumor angiogenesis and metastasis by gastric cancer. In the present study, we further examined whether and/or how NDRG1 could play any role in tumor angiogenesis by human gastric cancer cells. In light of the functional role of NDRG1 in gastric cancer cells, we discuss its significance in association with inflammatory stimulation and angiogenesis by gastric cancer cells.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Lines**—Human gastric cancer cell line NUGC-3 was purchased from Health Science Research Resources Bank. 58As1 and NCI-N87 were kindly provided by Dr. Kazuyoshi Yanagihara (National Cancer Center Research Institute, Tokyo, Japan) and Dr. Isamu Okamoto (Kyushu University, Fukuoka, Japan), respectively. As1/Mock3, As1/sic50, and As1/sic54 were established as described previously (24). The anti-NDRG1 antibody was generated as described previously (9). The anti-β-actin and anti-IL-1 receptor type 1 antibodies were purchased from Abcam; the anti-IL-1α, anti-c-Fos, anti-c-Jun, anti-Fra-1, anti-JunD, and anti-stable protein 1 (Sp-1) antibodies were from Santa Cruz Biotechnology; the anti-phosphorylated-Akt (phospho-Akt), anti-Akt, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-SAPK/JNK, anti-SAPK/JNK, anti-phospho-p38, anti-p38, anti-phospho-GSK3β, anti-GSK3β, anti-phospho-c-Jun, anti-c-Jun, anti-p65, anti-p105/p50, anti-p100/p52, anti-RelB, and anti-cAMP-response element-binding protein (CREB) antibodies were from Cell Signaling Technology; the anti-α-tubulin antibody was from Sigma; the anti-CD31 antibody was from BMA Biomedicals; and the anti-F4/80 was from AbD Serotec.

**Expression Vector Construction and Transfection**—Preparation of theFLAG-tagged NDRG1 expression plasmid was as described previously (21). Cells were transfected with FLAG-NDRG1 or FLAG-mock vectors by using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. Stable transfected clones were established by using G418 selection according to our previously published study (20). Preparation of the IL-8 promoter luciferase vector was as described previously (25).

**Gene Expression Microarray and Data Analysis**—Complementary RNA was amplified, labeled, and hybridized to a 44K Agilent 60-mer oligomicroarray according to the manufacturer’s instructions. All hybridized microarray slides were scanned by an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1). Raw signal intensities and flags for each probe were calculated from hybridization intensities (gProcessedSignal), and spot information (gIsSaturated), according to the procedures recommended by Agilent. Raw signal intensities of two samples were log_2-transformed and normalized using a quantile algorithm with a “preprocessCore” library package (26) on Bioconductor software (27, 28). To identify genes that were up-regulated or down-regulated, we calculated Z-scores and ratios (non-log scaled fold-changes) from the normalized signal intensities of each probe for comparison between control and experimental samples. We established criteria for up-regulated genes, Z-score ≥ 2.0 and ratio ≥ 2.0-fold; and down-regulated genes, Z-score ≤ −2.0 and ratio ≤ 0.5.

**Western Blot Analysis**—Cells were rinsed with ice-cold PBS and lysed in buffer containing 50 mmol/liter of Tris-HCl, 350 mmol/liter of NaCl, 0.1% Nonidet P-40, 5 mmol/liter of EDTA, 50 mmol/liter of NaF, 1 mmol/liter phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin, and 1 mmol/liter of Na3VO4. Cell lysates were subjected to Western blotting as described previously (20). The intensity of luminescence was quantified by using a charge-coupled device camera combined with an image-analysis system (LAS-1000; Fuji Film).

**Mouse Dorsal Air-sac Assay**—The assay was carried out in male mice at 7–10 weeks of age, as previously described (29, 30). Male BALB/c mice were obtained from Kyudo Co., Ltd. (Fukuoka, Japan). Cells (2 × 10⁶) were suspended in 150 μl of PBS and injected into a chamber that consisted of a ring covered with Millipore filters (0.45-μm pore size) on each side. On day 5, the chambers were removed from the subcutaneous fascia and replaced with black rings with the same inner diameter as the chambers. Photographs of these sites were assessed by counting the number of newly formed vessels that were more than 3 mm in length within the area of the rings.
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Collagen Gel Invasion Assay—Gastric cancer cells (6 × 10⁶) in serum-free RPMI 1640 were seeded onto cell–culture inserts (8-µm pore size; Falcon), coated with collagen gel, and 10% fetal bovine serum was added as a chemoattractant. After a 24-h incubation, cells on the upper surface of the filters were removed with a cotton swab, and the filters were fixed with 100% methanol and stained with Giemsa dye. The cells that had invaded the lower side of the filters were viewed under an Olympus microscope and counted in four fields of view. The assay was carried out as three independent experiments.

Matrigel Plug Assay—Mice were injected subcutaneously at the abdominal midline with 0.5 ml of growth factor-reduced Matrigel matrix (BD Bioscience) supplemented with NU/Mock and NU/Cap cells (2 × 10⁶). After 10 days, Matrigel plugs were removed, fixed with 10% buffered paraformaldehyde, and embedded in paraffin. Sections were stained with anti-CD31, anti-F4/80, hematoxylin and eosin. In therapeutic experiments with IL-1ra, 0.5 ml of growth factor-reduced Matrigel matrix containing IL-1ra (1 µg/ml) were also subcutaneously implanted into male KSN/sc nude mice (one Matrigel per mouse). Mice were killed 10 days later, and their Matrigel plugs were collected and processed as above.

ELISA—The concentrations of IL-1α, IL-8/CXCL8, VEGF-A, melanoma growth-stimulating activity α (GROα/CXCL1), and epithelial-derived neutrophil activating protein-78 (ENA78)/CXCL5 in the conditioned medium were measured by using commercially available ELISA kits (R&D systems), as described previously (20).

Luciferase Assay—pRL vector was cotransfected along with a firefly luciferase expression vector. After 24 h, the luciferase activity was measured according to the manufacturer’s instructions (Promega).

Chromatin Immunoprecipitation Assay—The chromatin immunoprecipitation (ChIP) assay was carried out using the EZ ChIP kit (Millipore Corp.), according to the manufacturer’s recommendations. Briefly, soluble chromatin was incubated with anti-c-Fos or c-Jun antibody. Purified DNA was used for PCR analysis with the following primer pairs: IL-8, 5’-GAAAATTTCTGT-CATACTCG-3’ (forward) and 5’-GAAAGTTTGCTGCT-TATGGAG-3’ (reverse); VEGF-A, 5’-GAGAGAAACCCCTATTTCCT-3’ (forward) and 5’-AGATGTTGCCAAGGAA-CTGA-3’ (reverse). PCR products were then analyzed on 2% agarose gels and stained with ethidium bromide.

**RESULTS**

Gastric Cancer Cell Expressing NDRG1 Has High Angiogenic Activity—We initially examined the expression of NDRG1 in three human gastric cancer cell lines (Fig. 1A). Of the three gastric cancer cell lines, expression of NDRG1 was found to be much higher in 58As1 than the other two cell lines, NCI-N87 and NUGC-3. We next compared angiogenic activity among three gastric cancer cell lines in vivo by using the dorsal air-sac assay. Implantation of chambers containing cancer cells resulted in the development of microvessels with thin curled structures and tiny bleeding spots, in addition to the preexisting vessels (Fig. 1B). There was significantly higher development of tumor angiogenesis by 58As1 cells than NCI-N87 or NUGC-3 cells (Fig. 1B), suggesting that NDRG1 may influence tumor angiogenesis by gastric cancer cell.

As1/sic50 and As1/sic54 cell lines were established by transfection of NDRG1 shRNA in 58As1 (Fig. 1C) (24). We compared angiogenic activity between NDRG1-silenced cell lines and their counterpart in vivo, using the dorsal air-sac assay. NDRG1 silenced cell lines showed less angiogenic activity than their parental counterpart (Fig. 1D). Next, we selected NUGC-3, harboring relatively lower expression of NDRG1, and established two independent sublines with higher expression of NDRG1, NU/Cap12, and NU/Cap32 by transfecting with NDRG1 complementary DNA (Fig. 1E). NU/Cap12 and NU/Cap32 cells showed higher angiogenic activity than NU/Mock3 cells (Fig. 1F), which was quantified as a significant 2-fold higher development of microvessels (Fig. 1G).

We further examined the effect of NDRG1 on angiogenesis in vivo, using a Matrigel plug assay. In the mouse Matrigel plug assay, neovascularatures were developed within the gel on day 10 after the inoculation of cancer cells (Fig. 2A). Matrigel plugs containing NU/Mock3 cells were transparent and pink in color, whereas plugs containing NU/Cap12 and NU/Cap32 were turbid and red with many bleeding spots indicating highly angiogenic responses (Fig. 2A). Immunohistochemical analysis using anti-CD31 and anti-F4/80 antibody of Matrigel showed a markedly increasing number of neovessels (CD31 positive) and infiltrating proinflammatory cells such as macrophages (F4/80 positive cells) in Matrigel plugs containing NDRG1-overexpressing cells as compared with their parental counterpart (Fig. 2B). Quantitative analysis showed significant (*, *p < 0.05) increases in microvascular density and infiltrating number of macrophages (Fig. 2, C and D).

NDRG1 Enhances Expression of IL-1, MMP-1, CXC Chemokines, and VEGF-A, and Phosphorylation of JNK—We next identified genes that were differentially expressed between NU/Cap12 and NU/Mock3 cells using microarray analysis (supplemental Table S1). In NU/Cap12 cells, 480 genes were up-regulated more than 2-fold by NDRG1, and 708 genes were down-regulated to less than 50% by NDRG1 (supplemental Table S1). The 480 up-regulated genes included 45 inflammation-related genes including angiogenic CXC chemokines (CCL1, -2, -3, -5, and -8), IL-1β, and IL-6 receptor (IL-6R). Representative inflammation-related genes are listed in Table 1.

We determined the expression levels of inflammation-related cytokines, angiogenic CXC chemokines, VEGF-A, and MMP-1 in NU/Cap12, NU/Cap32, and NU/Mock cells by ELISA or quantitative RT-PCR. Expression of the representative inflammatory cytokine IL-1α was about 2-fold higher in NU/Cap12 and NU/Cap32 cells than in NU/Mock3 (Fig. 3A). IL-1α is mainly a non-secretory cytokine (31), but there was a small amount of IL-1α secreted from NUGC-3 and its NDRG1-overexpressing cell lines. Although microarray analysis showed up-regulation of IL-1β (Table 1), ELISA revealed no detectable amounts of IL-1β protein in these three cell lines (data not shown). They showed much higher expression of three CXC chemokines, GROα/CXCL1, ENA-78/CXCL5, and IL-8/CXCL8, and VEGF-A than their parental counterpart (Fig. 3A). Expression of MMP-1 mRNA was also augmented in NU/Cap12 and NU/Cap32 cells (Fig. 3A). Western blot analysis
showed up-regulation of IL-1α expression by NDRG1 overexpression with similar expression levels of IL-1R type 1 to their parental counterpart (Fig. 3B).

Because the mRNA level of MMP-1 was found to be higher in NDRG1-overexpressing cell lines than its mock transfectant, we compared invasion activities in collagen gel. Fig. 3C shows marked differences between NU/Mock3 and NU/Cap12 or NU/Cap32 cells. Treatment with a MMP-1 inhibitor (GM-1489) suppressed invasion activity to 50–60% of control by both NU/Cap12 and NU/Cap32 cells (Fig. 3D), suggesting a partial involvement of MMP-1.

Liu et al. (17) have reported altered expression of ATF3 in prostate cancer cells by NDRG1 overexpression, and they also reported modification of the Wnt/β-catenin signaling pathway by NDRG1 in prostate cancer cells (18). We compared expression levels of several growth factor receptors, their downstream regulatory molecules, and Wnt/β-catenin-related proteins between NU/Mock3 and its NDRG1-overexpressing cell lines (Fig. 3E). We observed no difference in the expression of epidermal growth factor receptor, human epidermal growth factor (HER)2, HER3, MNNG-HOS transforming gene (c-Met), insulin-like growth factor-1R, pAkt, Akt, phosphatase and tensin homolog deleted on chromosome 10, mammalian target of rapamycin, and pAkt between NU/Cap12 and NU/Mock3 (data not shown). Furthermore, there was no apparent change in expression of β-catenin, pβ-catenin, E-cadherin, GSK-3β,
and pGSK-3β (Fig. 3E). However, expression and phosphorylation of JNK, a MAPK signaling molecule, were found to be augmented in NU/Cap12 and NU/Cap32 cells compared with NU/Mock3 (Fig. 3F). Phosphorylation and expression of other MAPK signaling molecules such as p38 and ERK were similar between NDRG1 transfectants and their parental counterpart (Fig. 3F).

**NDRG1 Induces Activation of AP-1**—We next investigated whether any transcription factor could be responsible for the observed NDRG1-dependent up-regulation of IL-1α, CXC chemokines, VEGF-A, and MMP-1. Western blot analysis showed no marked change in expression levels of NF-κB (p50/p65) and Sp-1 between NU/Mock3 and its NDRG1-overexpressing cell lines with total cell lysates (data not shown). Of various transcriptional factors being tested, expression of c-Fos and c-Jun was augmented in NU/Cap12 and NU/Cap32 compared with NU/Mock3 (Fig. 4A). By contrast, there was only a slight increase in expression of JunD and Fra-1 in NU/Cap12 and NU/Cap32 as compared with NU/Mock3. We further compared expression of AP-1, Sp-1, and NF-κB in the nucleus and cytoplasm. Nuclear expression of c-Fos and c-Jun was increased in both NU/Cap12 and NU/Cap32 cells compared with NU/Mock3 (Fig. 4B). However, there were no apparent changes in expression of p65, p50, RelB, or Sp-1 among cell lines.

We observed enhanced expression of c-Jun (Fig. 3F), and treatment with an inhibitor of JNK, SP600125, markedly blocked c-Jun phosphorylation in NDRG1-overexpressing cells (Fig. 4C). We next compared the promoter activity of AP-1 and NF-κB between NU/Mock3 and NU/Cap12 or NU/Cap32 cells (Fig. 4D). An AP-1-driven promoter luciferase assay showed 2.5–4.0-fold higher luciferase activity in NU/Cap12 and NU/Cap32 cells than their parental counterpart. There was only a 1.5–2-fold increase in NF-κB-driven promoter activity recorded between NU/Mock3 and NU/Cap12 or NU/Cap32 cells (Fig. 4D). NDRG1 overexpression thus mainly enhanced the AP-1-driven promoter activity, and slightly if any NF-κB-driven promoter activity.

We examined the effect of a NF-κB inhibitor SN50 on expression of IL-1α in NU/Cap12 cells (Fig. 4E). Expression of IL-1α was not suppressed by treatment with SN50. The ChIP assay revealed interaction of c-Jun and c-Fos with the promoter region of both IL-8/CXCL8 and VEGF-A (Fig. 4F). We next examined whether AP-1 was involved in IL-8/CXCL8 expression in NU/Cap12 and NU/Cap32 cells. IL-8/CXCL8 promoter

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**TABLE 1**

Selected inflammation-related genes up-regulated (more than 2-fold change) in NU/Cap12 cells versus NU/Mock3

| Gene symbol | Description | Ratio |
|-------------|-------------|-------|
| NDRG1       | N-myc downstream regulated gene 1 | 95.55 |
| CXCL1       | Chemokine (C-X-C motif) ligand 1 | 2.54 |
| CXCL2       | Chemokine (C-X-C motif) ligand 2 | 2.86 |
| CXCL3       | Chemokine (C-X-C motif) ligand 3 | 4.24 |
| CXCL5       | Chemokine (C-X-C motif) ligand 5 | 3.35 |
| CXCL8       | Chemokine (C-X-C motif) ligand 8 | 5.12 |
| IL-1β       | Interleukin 1β | 3.52 |
| IL-1F7      | Interleukin 1 family member 7 (ξ) | 2.51 |
| IL-6R       | Interleukin 6 receptor | 6.79 |
activity was significantly decreased by mutation of the AP-1 site in NU/Cap12 and NU/Cap32 (Fig. 4G).

The Regulatory Role of AP-1 in NDRG1-dependent Enhanced Expression of IL-1α, VEGF-A, CXC Chemokines, and MMP-1—We determined whether AP-1 could play critical roles in enhanced expression of IL-1α, VEGF-A, CXC chemokines, and MMP-1 by NDRG1 overexpression. Treatment with siRNAs for c-Jun or c-Fos resulted in apparent reduction of IL-1α protein expression in both NDRG1-overexpressing cell lines. Treatment with c-Jun siRNA also resulted in significantly reduced expression of IL-1α in NU/Cap12 and NU/Cap32 cells (Fig. 5A). Because IL-1α is mainly expressed as a non-secretory cytokine (31), we determined the effect of AP-1 knockdown on expression of IL-1α by Western blot analysis (Fig. 5, B and C). Treatment with c-Jun or c-Fos siRNA resulted in apparent reduction of IL-1α protein expression in both NDRG1-overexpressing cell lines. Treatment with c-Jun siRNA also resulted in significantly reduced expression of GROα/CXCL1, ENA-78/CXCL5, and IL-8/
CXCL8 in both NU/Cap12 and NU/Cap32 cells (Fig. 5D).

Treatment with c-Fos siRNA reduced expression of VEGF-A and IL-8/CXCL8 in both NU/Cap12 and NU/Cap32 cells. Expression of MMP-1 mRNA was significantly reduced by c-Jun siRNA, but not by c-Fos siRNA, in all three cell lines (Fig. 5E).

IL-1ra Suppressed NDRG1-induced Up-regulation of CXC Chemokines, VEGF-A, and MMP-1—Representative inflammatory cytokines, IL-1α or IL-1β, often promote expression of various cytokines/chemokines and growth factors in cancer cells and inflammatory cells (29, 30). We examined whether increased production of CXC chemokines and other cytokines could be mediated through autocrine control of IL-1α in NU/Cap12 and NU/Cap32 cells. Expression of VEGF-A, GROα/CXCL1, ENA-78/CXCL5, IL-8/CXCL8, and MMP-1 was significantly blocked by IL-1ra in NU/Cap12 and NU/Cap32 (Fig. 6, A and B). IL-8/CXCL8 promoter activity was also significantly blocked by IL-1ra in NU/Cap12 and
Expression of c-Jun rather than c-Fos in total cell lysates was specifically reduced when treated with IL-1ra (Fig. 6D). IL-1ra also inhibited phosphorylation of JNK and c-Jun (Fig. 6D). Nuclear expression of c-Jun was also markedly blocked by IL-1ra, but there was no apparent change in nuclear expression of c-Fos (Fig. 6E). We also confirmed that nuclear expression of p50 and p65 was also blocked by IL-1ra.

**IL-1ra Suppressed Tumor Angiogenesis and Macrophage Infiltration**—We finally examined the therapeutic effect of IL-1ra on tumor angiogenesis by Matrigel plug assay. Neovasculatures were developed within the Matrigel on day 10 after the inoculation of NU/Cap12 cells, whereas treatment with IL-1ra markedly suppressed the development of neovasculatures (Fig. 7A). Immunohistochemical analysis using anti-CD31 and anti-F4/80 antibodies of Matrigel showed an increasing number of neovessels and infiltrating macrophages in the Matrigels containing NU/Cap12 cells (Fig. 7, B and C) (see also Fig. 2). By contrast, there was a decreasing number of both neovessels and infiltrating macrophages when treated with IL-1ra (Fig. 7, B and C). Quantitative analysis of 8 Matrigel plugs showed significant (**, p < 0.01) decreases in both microvessel density and number of infiltrating macrophages by IL-1ra treatment (Fig. 7D).
DISCUSSION

Our present study demonstrated novel roles of NDRG1 in gastric cancer cells as follows: 1) much higher angiogenic activity together with infiltration of macrophages by NDRG1 was demonstrated in a tumor angiogenesis assay in vivo; 2) overexpression of NDRG1 induced up-regulation of angiogenic CXC chemokines, VEGF-A, and MMP-1; 3) cell invasion and enhanced MMP-1 expression, but not cell growth, were markedly stimulated by NDRG1; 4) JNK phosphorylation was upregulated by NDRG1; 5) expression of the representative inflammatory cytokine IL-1α was up-regulated by NDRG1; 6) expression and phosphorylation of AP-1 (Jun/Fos) were up-regulated by NDRG1 and this activation was suppressed by an antagonist of IL-1α (IL-1ra); 7) treatment with IL-1ra markedly suppressed tumor angiogenesis and infiltration of macrophages in response to NDRG1 in vivo. Based on the above results, in gastric cancer cells, NDRG1 thus seems to induce up-regulation of IL-1α, a representative inflammatory cytokine, and enhanced expression of angiogenic growth factors, CXC chemokines, and matrix metalloproteinase, possibly through JNK/AP-1-induced activation of IL-1α-driven signaling pathway, resulting in tumor angiogenesis (Fig. 7E).
**FIGURE 7.** Therapeutic effect of IL-1ra on tumor angiogenesis and infiltration of macrophages by gastric cancer cells. 

_A_, photographs of Matrigel plug containing NDRG1 transfectant NU/Cap12 with or without IL-1ra treatment. Four plugs for each control and treated group are presented. 

_B_, tumor angiogenesis in Matrigel containing NU/Cap12 with or without IL-1ra treatment. Neovessels (CD31) in the Matrigel plugs were immunostained; arrowhead, CD31 positive neovessels in 4 plugs. 

_C_, macrophages infiltrating in Matrigel containing NU/Cap12 with or without IL-1ra treatment. Macrophages (F4/80) in Matrigel were immunostained; F4/80 positive macrophages in four plug samples are shown. 

_D_, quantitative analysis of newly formed blood vessels and infiltrating macrophages in Matrigel plugs containing NU/Cap12 with or without IL-1ra treatment. Each column was average of 8 Matrigel plugs (**, p < 0.01) versus control. 

_E_, our hypothetic model how NDRG1 promotes tumor angiogenesis in its close context with IL-1α by gastric cancer cells. In this study, human gastric cancer cells overexpressing NDRG1 induces enhanced expression of the inflammatory cytokine IL-1α through activation of JNK and AP-1 (Jun/Fos), resulting in promoted expression of angiogenesis-related factors affecting tumor angiogenesis.
The ability of NDRG1 to act as a predictive marker for cancer patients depends upon tumor types (1, 2), suggesting that NDRG1 has promotive or suppressive effects on tumor growth and metastasis in close context with specific malignant changes in each tumor. In gastric cancer, NDRG1 expression was found to be predictive for malignant progression, poor prognosis, and tumor angiogenesis (22, 23). Consistent with these clinical studies, we have recently reported that highly metastatic human gastric cancer cell lines showed relatively higher levels of NDRG1 expression than their low metastatic counterpart (24). NDRG1 knockdown in this highly metastatic gastric cancer cell increased expression of E-cadherin through decreased expression of Snail, indicating that NDRG1 promotes metastasis and growth of the aggressive gastric cancer cell through epithelial mesenchymal transition (24). However, Wnt/β-catenin signaling pathway was not involved in NDRG1-induced epithelial mesenchymal transition by gastric cancer cells (24). In our present study, we observed tumor angiogenesis and infiltration of macrophages by NDRG1 overexpression in gastric cancer cells when determined by the dorsal air-sac assay and the Matrigel plug assay (Figs. 1, 2, and 7). Tumor angiogenesis is often associated with malignant progression of gastric cancer (32), indicating that NDRG1 may promote tumor growth and metastasis by gastric cancer cells. Furthermore, infiltration and activation of macrophages such as tumor-associated macrophages may provide a tumor microenvironment favorable condition for malignant progression of cancer cells (33, 34).

NDRG1-induced angiogenesis is found to be blocked by treatment with IL-1ra (Fig. 7D), indicating that IL-1 plays important roles in angiogenesis by NDRG1. NDRG1 overexpression also induces increasing expression of VEGF-A, CXC chemokines, and MMP-1 as well as IL-1α in gastric cancer cells (Fig. 3). This NDRG1-induced higher expression of such angiogenesis-related factors is blocked when treated with IL-1ra, indicating again that NDRG1-driven up-regulation is mainly due to IL-1-induced angiogenic signaling (Fig. 7E). Furthermore, IL-1/IL-1R has been often known to activate various cellular signaling pathways including mTOR, MAPK, NF-κB, Sp-1, and AP-1 (31, 35). JNK, a member of the MAPK family, phosphorylates and activates c-Jun (36). Expression and phosphorylation of JNK and AP-1 were up-regulated by NDRG1 in the present study (Figs. 3F and 4, A and B). Furthermore, it has been reported that transcription of VEGF-A and IL-8/CXCL8 was regulated by AP-1 (37, 38). Consistent with these reports, c-Jun and c-Fos bound to VEGF-A and the IL-8 promoter, and IL-8 promoter activity was markedly suppressed by mutation of the AP-1 site, and also by IL-1ra in NDRG1 transfectant (Figs. 4, F and G, and 6C).

Previously studies have reported that NDRG1 suppresses ATF3 expression and ATF3NF-κB complex formation through inhibition of the Wnt/β-catenin signaling pathway in prostate cancer cells (17, 18). However, in our present study, expression of ATF3, NF-κB, and β-catenin is not markedly different between NU/Mock3 and its NDRG1 transfectant. The NF-κB-driven promoter shows only a slight activation in NDRG1 transfectants than their parental counterpart. The activated AP-1, mostly Jun, induces expression of VEGF-A, MMP-1, and also angiogenic CXC chemokines that have chemotactic activity for neutrophils and monocytes/macrophages when NDRG1 is overexpressed in human gastric cancer cell. NDRG1 may thus stimulate tumor stromal responses including angiogenesis and cell motility through transcriptionally positive regulation by AP-1, probably by Jun, in gastric cancer. Further study should be required to understand how NDRG1-induced expression of such CXC chemokines plays important roles in the recruitment of macrophages and angiogenesis.

The IL-1 family consists of two major agonistic proteins: IL-1α and IL-1β, which exert their inflammatory and immunological activities through interaction with IL-1R (31). Although IL-1α and IL-1β show distinct roles in inflammation and immunity (31), both potent inflammatory cytokines induce an angiogenic switch, affecting invasion and metastasis by cancer cells (31, 33). We have previously reported that the inflammatory cytokine-induced angiogenesis was mediated through up-regulation of angiogenic factors, inflammatory cytokines, chemokines, and MMPs not only in cancer cells but also in tumor stromal cells including tumor-associated macrophages (35, 39–41). In particular, IL-1-driven angiogenesis was often accompanied by infiltration and accumulation of tumor-associated macrophages that produced potent angiogenic growth factors and CXC chemokines, supporting further development of new blood vessels. Consistent with these studies, we also observed increased accumulation of macrophages in Matrigel plugs containing NU/Cap12 cells expressing IL-1α (Figs. 2 and 7), and treatment with an IL-1ra markedly blocked expression of CXC chemokines in vitro (Fig. 6) and accumulation of macrophages in vivo (Fig. 7, C and D). In human gastric cancer cells, IL-1α is abundantly produced and promotes the expression of several growth factors through its autocrine loop (42). Furthermore, stomach-specific expression of IL-1β in transgenic mice leads to spontaneous gastric inflammation and cancer (43). Together with our present study, it seems likely that the inflammatory cytokine IL-1α plays an important role in inflammation and angiogenesis by gastric cancer, possibly through infiltration and activation of macrophages in close association with NDRG1. In conclusion, NDRG1 plays essential roles in tumor angiogenesis by gastric cancer through IL-1-induced inflammatory stimuli. NDRG1 may be a useful target for the development of novel molecular diagnosis and therapeutic strategy against inflammation involving angiogenesis and invasion by gastric cancer. Our findings shed light on how the IL-1/IL-1R signaling pathway could support angiogenesis and malignant progression by gastric cancer cells in context with NDRG1.

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