All-trans-Retinoic Acid Induces Nuclear Factor κB Activation and Matrix Metalloproteinase-9 Expression and Enhances Basement Membrane Invasivity of Differentiation-resistant Human SK-N-BE 9N Neuroblastoma Cells

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Abstract
A comparison between retinoic acid (RA) differentiation-resistant and differentiation-sensitive SK-N-BE neuroblastoma (NB) cell lines revealed an association between resistance to differentiation, exhibited by N-myc stable transfected SK-N-BE 9N cells, with sensitivity to RA induction of p50/p65 nuclear factor κB (NF-κB) transcription factor activity and induction of matrix metalloproteinase (MMP)-9 expression leading to enhanced invasive behavior in vitro. These effects were not observed in differentiation-sensitive parental SK-N-BE or control-transfected SK-N-BE 2N counterparts. RA activated a MMP-9 promoter reporter gene construct in SK-N-BE 9N but not parental SK-N-BE or SK-N-BE 2N cells through a NF-κB element (−600) in association with enhanced p50 mRNA expression, reduced cytoplasmic inhibitor of nuclear factor κBα protein levels, and the induction of nuclear p50/p65 containing MMP-9 NF-κB site binding activity. RA activation of the MMP-9 promoter was inhibited by transient overexpression of a dominant-negative inhibitor of nuclear factor κBα protein and stimulated by transient p50 but not p65 overexpression in the absence of RA. A limited, nonessential function for activator protein 1 (−74), Ets (−540), and SP1 (−560) elements within the MMP-9 promoter was revealed by point mutation but was not associated with changes in the binding or position of complexes constitutive to differentiation-sensitive or -resistant cells. Our data indicates that in this model of NB resistance to differentiation that results from uncoupled RA regulation of N-myc expression, RA stimulates malignant NB cell behavior by inducing nuclear NF-κB transcription factor activity, which in turn induces MMP-9 expression and stimulation of basement membrane invasive capacity involving MMP-9 activity.

Introduction
NB is a highly malignant childhood tumor of neural crest origin, a sizeable proportion of which exhibits amplification and endogenous activation of the proto-oncogene N-myc, the amplification and expression of which have been closely associated with aggressive and advanced stage disease and therefore to a more malignant NB phenotype (1–4).

N-myc is a member of the myc transcription factor family (5), which in addition to its association with NB progression, is a critical determinant in the capacity of NB cells to terminally differentiate in response to derivatives of RA, currently used in adjuvant differentiation-based therapy of residual and/or relapsed NB (1, 6–9). RA-mediated terminal differentiation of N-myc-amplified NB cells is considered to depend upon transient down-regulation of N-myc expression through RA responsive elements within the N-myc promoter (7–9). Indeed, the uncoupling of this regulatory mechanism resulting from mutations within responsive elements (10) or by overexpression of exogenous N-myc (8) promotes a differentiation-resistant phenotype considered to represent one mechanism by which NBs can develop a RA differentiation-resistant phenotype in vivo.

RA differentiation of NB cells has been reported to associate with NF-κB transcription factor activity (11) or not to involve this factor (12). NF-κB transcription factors are comprised of heterodimers between members of the rel family that include RelA, Rel B, p50, and p65 protein. NF-κB activity is regulated by de novo component synthesis, nuclear p50 translocation, which is regulated by changes in the level of cytoplasmic I-κB proteins which retains p50/p65 heterodimers within the cytoplasm and by redox conditions within the nucleus (13, 14). NF-κB transcription factor components, including p50 and p65, are regulated by RA and

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3 The abbreviations used are: NB, neuroblastoma; RA, retinoic acid; NF-κB, nuclear factor κB; I-κB, inhibitor of nuclear factor-κB; MMP, matrix metalloproteinase; AP-1, activator protein 1; BM, basement membrane; CAT, chloramphenicol acetyltransferase; RAR, retinoic acid receptor; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TIMP, tissue inhibitor of metalloproteinase; EMEM, Eagle minimal essential medium.
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N-myc, with N-myc reported to down-regulate p50 expression (11, 15–17).

Malignant tumor progression depends upon the capacity to invade, metastasize, and promote an angiogenic host response. Among the many gene products involved in this process are members of the MMP family, several of which are overexpressed by malignant tumors and exhibit tumor invasion and angiogenesis-promoting activity (18). Within the MMP family, attention has focused on MMP-9, a Mr 92,000 gelatinolytic/type IV collagenolytic metalloproteinase implicated in the degradation of BM associated with tumor invasion, in the induction and maintenance of the malignant phenotype and the promotion of angiogenesis (18–25). MMP-9 is overexpressed in advanced stage metastatic NBs (26, 27) in other human malignancies and by malignant cell lines, including non-N-myc-amplified NB cell lines (28–31), and MMP-9 expression can be induced by oncogenes, inflammatory cytokines, growth factors, and protein kinase-C-activating phorbol esters (18, 28–30, 32–35). In contrast to non-N-myc-amplified NB cells, N-myc-amplified NB cell lines do not constitutively express MMP-9 in vitro (2), suggesting that MMP-9 involvement in N-myc-amplified NB in vivo would depend upon the induction of its expression by tumor and/or nonmalignant elements (26, 27).

The 5′ flanking region of the MMP-9 gene has been cloned and shown to possess constitutive and inducible promoter activity when linked to reporter genes (18, 22, 31, 33–39). Sequence analysis has revealed several potential regulatory regions involved in basal and induced MMP-9 transcriptional responses. An AP-1 element at position −79 relative to the transcriptional start site is necessary but insufficient for phorbol ester and cytokine-enhanced MMP-9 transcription in fibrosarcoma cells and cooperates with NF-κB and Sp1 elements at positions −600 and −560, respectively (22). The NF-κB element (−600) is involved in Bcl-2 induction of MMP-9 transcription in breast cancer cells (39). The Ets elements at position −571 and −540 have been implicated in MMP-9 promoter activation by Ras (33), E1AF (36), fibroblast cell contact (37), and by epidermal growth factor (38) and shown to possess constitutive and inducible promoter activity when linked to reporter genes (18, 22, 31, 33–35). In contrast to non-N-myc-amplified NB cells, N-myc-amplified NB cell lines do not constitutively express MMP-9 in vitro (2), suggesting that MMP-9 involvement in N-myc-amplified NB in vivo would depend upon the induction of its expression by tumor and/or nonmalignant elements (26, 27).

The results of this study, therefore, have used models of RA differentiation-sensitive and -resistant NB, exemplified by the differentiation-sensitive parental SK-N-BE and control-transfected SK-N-BE 2N cells lines and differentiation-resistant, stable, N-myc-transfected SK-N-BE 9N cell line (8), to study the association between resistance and sensitivity to RA-mediated differentiation, NF-κB transcription factor activation, MMP-9 transcription, and BM invasive behavior in vitro as an index of malignant behavior. We report an association between differentiation resistance, exhibited by SK-N-BE 9N cells, and sensitivity to induction of NF-κB transcription factor activity, NF-κB-mediated MMP-9 expression, and to stimulation of BM invasive capacity of differentiation-resistant cells, which involve MMP-9 activity.

Results

RA Terminally Differentiates SK-N-BE and SK-N-BE 2N but not SK-N-BE 9N. In confirmation of previous reports (8, 41), RA (1 and 10 μM) induced ganglioneuronal differentiation of parental SK-N-BE and control-transfected SK-N-BE 2N cells but did not differentiate SK-N-BE 9N cells stable transfected with an exogenous N-myc gene (Fig. 1A, photomicrographs displayed for 0 and 15 days of RA (1 μM) treatment only). Neurite extension induced by RA in parental and 2N cells could be detected within 3 days and was followed by cell migration to form interconnected ganglion-like structures between 10 and 15 days (Fig. 1A). RA neither induced neurite extension nor ganglion-like structure formation by SK-N-BE 9N cells over the same time course (Fig. 1A). RA (1 μM) reduced N-myc mRNA levels at 24 h and N-myc protein levels by 24 and 48 h in SK-N-BE and SK-N-BE 2N cells but did not reduce either N-myc mRNA or protein levels expressed by SK-N-BE 9N cells (Fig. 1B).

CAT assays revealed similar levels of RA-mediated tk-
TREp2CAT induction in SK-N-BE, SK-N-BE 2N, and SK-
N-BE 9N cells after transient transfection, indicating that all three cell lines were RA responsive at the level of RAR element-mediated transcriptional activation (Fig. 1C). All three cell lines constitutively expressed retinoid X receptor, RARα, β, and γ mRNA, as determined by Northern blotting (data not displayed).

RA Induces NF-κB Activity in SK-N-BE 9N but not SK-
N-BE or SK-N-BE 2N Cells. NF-κB transcription factor activity has been associated with, or reported not to be involved, in the differentiation response of NB cell lines to RA (11, 12). In this study, using a MMP-9 NF-κB element oligonucleotide, we observed RA (1 and 10 μM) induction of specific nuclear NF-κB site binding complexes in differentiation-resistant SK-N-BE 9N but not in SK-N-BE or SK-N-BE 2N cells assayed 1, 3, 6, and 9 days after initiation of RA treatment [Fig. 2A, data are displayed for SK-N-BE 2N and SK-N-BE 9N cells at 3 days of RA (1 μM) treatment]. Spec-
ficity of binding was confirmed by competition EMSA assay in which cold-specific oligonucleotide, but not cold MMP-9 AP-1 (used as a nonspecific oligonucleotide) or point-mutated NF-κB oligonucleotides, competed with the labeled probe (Fig. 2A). Supershift assays were used to characterize the protein composition of NF-κB site binding complexes using specific antibodies previously reported to supershift DNA binding complexes containing the relevant proteins. Supershift assays confirmed the presence of both p50 and p65 but not cRel proteins in NF-κB site binding complexes induced by RA in SK-N-BE 9N cells (Fig. 2B).

Transcriptional function of the NF-κB complexes induced by RA in SK-N-BE 9N cells was confirmed by CAT assay in which RA activated a CAT reporter gene bearing a single NF-κB element (Δ56NF-κBCAT), but not its corresponding control vector, in SK-N-BE 9N but not SK-N-BE or SK-N-BE 2N cells (Fig. 2C, data are displayed for Δ56NF-κBCAT reporter gene only).

RA Enhances p50 mRNA Expression and Reduces IκBα Protein Levels in SK-N-BE 9N Cells. The induction of NF-κB transcription factor activity in SK-N-BE 9N cells was associated with an increase in the levels of p105/50 mRNA by ~2.5-fold with respect to GAPDH levels 3 days after RA treatment, reducing thereafter (Fig. 3A). Constitutive P65 mRNA levels were minimally modulated by RA. A ~2-fold increase in constitutive IκBα mRNA levels, relative to GAPDH levels, was also detected in SK-N-BE 9N cells 6 and 9 days after RA treatment. RA did not significantly modulate p50, p65, or IκBα mRNA levels in either SK-N-BE or SK-N-BE 2N cells (Fig. 3A).

Western blots detected reduced cytoplasmic IκBα protein levels in SK-N-BE 9N cells 3, 6, and 9 days after RA treatment, whereas constitutive IκBα protein levels in SK-N-BE or SK-N-BE 2N cells were not reduced over the same time course (Fig. 3B). Actin protein levels, used as a control protein, were not modulated by RA in SK-N-BE 9N cells (data not displayed).

RA Induces Rapid MMP-9 Expression in SK-N-BE 9N but not SK-N-BE or SK-N-BE 2N Cells. Neither parental SK-N-BE, SK-N-BE 2N, nor SK-N-BE 9N cells constitutively express MMP-9. RA (1 and 10 μM) rapidly induced MMP-9 expression in 9N cells, which could be detected at low levels within 24 h (data not displayed), increasing thereafter to 15 days. MMP-2 levels were not affected by RA treatment (Fig. 4A, data are displayed for 1 μM RA only). MMP-2 and MMP-9 identity was confirmed by Western blot (Fig. 4B). RA induced MMP-9 mRNA expression in SK-N-BE 9N cells, as determined by Northern blot (Fig. 4C). In contrast to effects on 9N cells, RA (1 and 10 μM) induced only very low levels and delayed MMP-9 protein expression (Fig. 4A) without evidence of MMP-9 mRNA induction (Fig. 4C) in SK-N-BE and SK-N-BE 2N cells. Constitutive TIMP-1 and TIMP-2 protein
and mRNA levels were not stimulated by RA (1 and 10 μM) in either of the three cell lines (data not displayed).

The addition of an anticatalytic anti-MMP9 antibody (100 μg/ml; Ref. 42) to SK-N-BE 2N or SK-N-BE 9N cultures at days 10–15 of RA (1 μM) treatment neither impaired ganglioneuronal differentiation of SK-N-BE 2N cells nor induced morphological differentiation of RA-treated SK-N-BE 9N cells, as judged by phase contrast microscopy (Fig. 4).

RA Activates the MMP-9 Promoter in SK-N-BE 9N Cells through a NF-κB Element. A MMP-9 promoter CAT reporter gene construct bearing 670 bp of 5′ MMP-9 flanking region containing the major transcriptional elements (22, 31) transiently transfected into parental SK-N-BE, SK-N-BE 2N, and SK-N-BE 9N cells was activated by RA (1 and 10 μM) only by SK-N-BE 9N cells when assayed for up to 9 days of RA treatment [Fig. 5A, data are displayed for RA (1 μM) treatment of SK-N-BE 2N and 9N cells only, at 3 and 6 days]. Deletion (−586MMP-9) or point mutation (−670mMMP-9) of the NF-κB element (−600) within the MMP-9 promoter completely abrogated RA-mediated promoter activation in SK-N-BE 9N cells (Fig. 5, B and C). Individual point mutations of either Ets (−571), SP1 (−560), and AP-1 (−79) elements within the MMP-9 promoter all significantly (P < 0.001) inhibited RA activation of the MMP-9 promoter by between 30 and 40% in SK-N-BE 9N cells, with no single point mutation capable of abrogating RA responsiveness (Fig. 5B). Point mutation of a second Ets element at position −540 or the GT box (−49) within the MMP-9 promoter did not significantly inhibit RA-mediated promoter activation in SK-N-BE 9N cells (data not displayed for the Ets element). All mutated elements were characterized as unable to bind specific nuclear complexes in RA-treated nuclear extracts from SK-N-BE 9N cells, as judged by EMSA using oligonucleotides bearing the same point mutation (see “Materials and Methods”).

In addition to the absolute requirement for the NF-κB site in RA activation of the MMP-9 promoter, AP-1, Ets, and Sp1 function was associated with specific nuclear Ets, Sp1, and AP-1 site binding activity. However, in contrast to NF-κB site binding complexes, complexes binding to these other elements were found to be constitutive to all three cell lines and neither binding levels nor composition were significantly modified by RA (data not displayed). The composition of the constitutive complexes present at similar levels in SK-N-BE, SK-N-BE 2N, and SK-N-BE 9N cells were as follows: the AP-1 complex(es) in all three cell lines contained junD, fraI, and fraII but not cjun, junB, cfos, or fosB proteins; the Sp1 binding complex(es) in all three cell lines contained Sp2 and Sp3 but not Sp1 or Sp4 proteins; and the Ets binding complex(es) were not characterized (data not displayed).

p50 Activates and Dominant Negative I-κBα Inhibit RA-mediated Activation of the MMP-9 Promoter in SK-N-BE 9N Cells. Transient cotransfection of a dominant negative I-κBα mammalian expression vector (43), but not an empty vector control (pmT2T), into SK-N-BE 9N cells significantly
inhibited the induction of MMP-9 promoter activity by ∼50% (P < 0.001; n = 6) after treatment with 1 μM RA for 72 h (Fig. 6A). Transient cotransfection of SK-N-BE 9N cells with a P50 mammalian expression vector (44), but not empty expression vector control, resulted in the induction of MMP-9 promoter activity assayed at 72 h in the absence of RA. Promoter activity corresponded to ∼25% that observed in RA-treated non-cotransfected SK-N-BE 9N cells. Transfection of SK-N-BE 9N cells with p50 expression vector did not induce activation of the MMP-9 promoter construct mutated in the NF-κB element (−670mNF-κB; Fig. 6A). Cotransfection with a p65 expression vector (43) did not induce MMP-9 promoter activity in SK-N-BE 9N cells in the absence of RA.

In confirmation that changes in N-myc expression were responsible for the sensitization of SK-N-BE 9N cells to RA induction of MMP-9 promoter activity through the NF-κB element, transient transfection of SK-N-BE 2N cells with a N-myc expression vector, but not empty vector control, sensitized cells to RA (1 μM) activation of the intact −670MMP9 reporter gene construct but not its NF-κB mutated counterpart −670NF-κBm (Fig. 6B) and to activation of a reporter gene construct bearing a single copy of the MMP-9 NF-κB element (Δ56NF-κB; Fig. 6C). The function of the N-myc expression vector was confirmed by RT-PCR and Western blotting after transient transfection into non-N-myc expressing SH-EP NB cells (Fig. 6D).

RA Stimulates Matrigel Invasion of SK-N-BE 9N but not SK-N-BE or SK-N-BE 2N Cells. Treatment of parental SK-N-BE, SK-N-BE 2N, and SK-N-BE 9N cells for 6 days with RA (1 μM) before Matrigel invasion assay resulted in a significant (P < 0.001; n = 10) and ∼2-fold increase in SK-N-BE 9N but SK-N-BE or SK-N-BE 2N invasion (Fig. 7). RA slightly but not significantly reduced the invasive capacity of SK-N-BE and SK-N-BE 2N cells. An anticatalytic anti-MMP-9 antibody (100 μg/ml; Ref. 42) significantly (P < 0.02; n = 6) reduced RA stimulation of SK-N-BE 9N cell invasion by between 30 and 40% but did not inhibit basal SK-N-BE 9N invasion nor SK-N-BE and SK-N-BE 2N invasivity in the presence or absence of RA (1 μM; Fig. 7).

Discussion
The attainment of a differentiation-resistant phenotype by uncoupling RA regulation of N-myc expression is considered one mechanism whereby NBs may escape the differentiating effects of RA in vivo (1, 8, 10). Little is known, however, concerning the effects of continued RA responsiveness by differentiation-resistant NBs on malignant behavior in the
presence of RA. In this study, using a model of RA differentiation-sensitive NB rendered resistant to differentiation by exogenous N-myc gene expression, we report an association between differentiation resistance, induction of p50/p65 NF-κB transcription factor activity, rapid induction of high-level MMP-9 expression mediated by NF-κB, and subsequent stimulation of invasive capacity through reconstituted BM Matrigel in vitro involving MMP-9 activity.

Parental SK-N-BE and control-transfected SK-N-BE 2N cells terminally differentiated into interconnecting ganglion-like structures within 15 days of RA treatment. In contrast, stable N-myc-transfected SK-N-BE 9N cells were resistant to the terminal differentiating effects of RA, confirming previous reports (8, 41). Also consistent with the latter reports, RA reduced N-myc mRNA and protein expression in SK-N-BE and SK-N-BE 2N but not SK-N-BE 9N cells, supporting the hypothesis that down-regulation of N-myc expression is a prerequisite for the differentiating effects of RA (7, 10). Resistance to RA-mediated differentiation exhibited by 9N cells was not associated with a lack of RA responsiveness at the level of RAR element-mediated gene transcription because differentiation-sensitive and -resistant cells exhibited similar capacity to activate a reporter gene construct bearing a classical RAR element in response to RA and constitutively expressed retinoid X receptor, RAR α, β, and γ mRNAs.

RA differentiation of parental SK-N-BE and SK-N-BE 2N cells was not associated with induction of NF-κB transcription factor activity. However, RA induced NF-κB activity in differentiation-resistant SK-N-BE 9N cells. This adds to a previous report that RA differentiation of SK-N-BE cells is not associated with NF-κB activation (12) and is in contrast to the report role for NF-κB in RA-mediated terminal differentiation of the non-N-myc-amplified SH-SY5Y NB cells (11). Thus both NF-κB-dependent and -independent mechanisms would appear to exist for RA-mediated terminal differentiation, which may differentiate between N-myc-amplified and -nonamplified disease.

RA induction of NF-κB activity in SK-N-BE 9N cells was associated with stimulation of p105/50 but not p65 mRNA expression. Consistent with the reported capacity of N-myc to down-regulate p50 expression (15, 16), constitutive p50 mRNA levels were low in all three N-myc-amplified SK-N-BE cell lines. However, RA stimulated p50 expression in either parental SK-N-BE or SK-N-BE 2N cells despite reducing N-myc levels. This would suggest a more complicated relationship between RA, N-myc, and p50 expression that may involve changes in N-myc-associated factor X (MAX) ratios, not evaluated here.
but considered critical for the regulation of gene expression (8, 45).

In addition to stimulating p105/50 expression, RA stimulated mRNA levels of the cytoplasmic p50/p65 sequestator I-κBα in SK-N-BE 9N cells but not in parental SK-N-BE or SK-N-BE 2N cells, suggesting conditions unique to uncoupled RA/N-myc NB that regulate both p50 and I-κBα mRNA levels. However, in contrast to its effects on I-κBα mRNA, RA reduced cytoplasmic I-κBα levels of I-κBα protein in SK-N-BE 9N but not parental SK-N-BE or SK-N-BE 2N cells, further suggesting conditions unique to uncoupled RA/N-myc interactions that promote cytoplasmic I-κBα elimination. This apparently contradictory effect upon I-κBα mRNA and protein levels most likely reflects cytoplasmic elimination of I-κBα via the ubiquitin pathway, considered a major mechanism in permitting nuclear NF-κB translocation, which is independent of mRNA expression (43). Thus within the context of SK-N-BE 9N cells, RA, by increasing p50 expression and reducing I-κBα levels, appears to have used a mechanism widely implicated in the regulation of NF-κB activation (43). Although, how such a change in the N-myc/RA relationship causes this remains to be elucidated, the result was the induction of transcriptionally functional p50/p65 NF-κB activity. Confirmation that RA induction of NF-κB in SK-N-BE 9N cells was attributable to changes in N-myc expression and not peculiarities of a selected stable transfected cell line was obtained by transient transfection of an N-myc expression vector into SK-N-BE 2N cells, which resulted in sensitivity to RA activation of a reporter gene bearing a single NF-κB site in contrast to empty vector-transfected counterparts.
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Consistent with its capacity to induce NF-κB activity in SK-N-BE 9N but not in SK-N-BE or SK-N-BE 2N cells, RA also induced rapid high-level expression of the tumor invasion, metastasis, and angiogenesis-associated NF-κB-regulated gene MMP-9 (22, 31, 39) in SK-N-BE 9N but not the other cell lines. Furthermore, RA activated an MMP-9 promoter CAT reporter gene construct bearing the major transcriptional elements (22) in SK-N-BE 9N, but not SK-N-BE or SK-N-BE 2N cells, but failed to activate MMP-9 constructs in which the NF-κB element was mutated or had been deleted, confirming an absolute requirement for the NF-κB element. The delay in MMP-9 mRNA expression relative to detection of NF-κB activity and capacity to activate the MMP-9 promoter suggest additional posttranscriptional mechanisms in the regulation of MMP-9 expression consistent with a recent report (46).

A central role for p50 in RA-induced MMP-9 transcription in SK-N-BE 9N cells was confirmation using a p50 mammalian expression vector (44), which induced MMP-9 promoter activity in 9N cells in the absence of RA upon transient transfection. The role of reduced cytoplasmic IκBα levels in RA-induced MMP-9 transcription by SK-N-BE 9N cells was confirmed using a dominant negative mutated IκBα, insensitive to cytoplasmic degradation (43), which significantly inhibited RA activation of the MMP-9 promoter in SK-N-BE 9N cells.

In addition to the absolute requirement for the NF-κB element, RA induction of MMP-9 promoter activity in SK-N-BE 9N cells also involved AP-1 (–79), Ets (–571), and Sp1 (–560) elements. These elements bound specific nuclear complexes constitutive to all three cell lines, the composition and level of which were not altered by RA. These elements have all previously been reported to be involved in MMP-9 transcription (22, 33–37). We found no evidence of a necessary requirement for any one of these elements alone, in contrast to previous reports of an absolute but insufficient...
role for the AP-1 (−79) element for MMP-9 transcription (22). It is possible that Ets binding transcription factors such as E1A/F constitutive to N-myc-amplified NB cells (2), which exhibit the capacity to transcriptional activate the MMP-9 promoter independently of the AP-1 element (36, 37), may help to explain our observations. No function could be attributed to the GT box (−49) in RA activation of the MMP-9 promoter in SK-N-BE 9N cells. This element has previously been implicated in constitutive MMP-9 transcription by N-type, non-N-myc-amplified NB cells (31). Additional confirmation that RA effects on SK-N-BE 9N cells was not solely because of the peculiarities of a selected stable-transfected cell line but rather upon changes in RA/N-myc interactions was obtained using SK-N-BE 2N cells transiently transfected with an N-myc mammalian expression vector, but not empty vector transfected controls, which became sensitive to RA activation of the intact MMP-9 promoter reporter gene construct but not the MMP-9 promoter construct mutated within the NF-κB element.

The induction of MMP-9 expression has previously been associated with RA terminal differentiation of SK-N-BE cells and a role for MMP-9 implicated in the neuronal differentiation process (40). In this study, RA induced extremely low-level MMP-9 expression by SK-N-BE and SK-N-BE 2N cells in association with late stages of differentiation as detected by zymogram but not by Northern blotting. Furthermore, RA did not activate the MMP-9 promoter in either cell line nor was differentiation impaired by an anticytotoxic anti-MMP-9 antibody at MMP-9 inhibitory concentrations (31, 42). Taken together, the data suggest that MMP-9 is not required for terminal differentiation of either SK-N-BE or SK-N-BE 2N cells and that its expression is independent from the differentiation process. This is consistent with previous reports that MMP-9 expression associates with more malignant NB phenotypes in vitro and in vivo (27, 31). We found no evidence, however, that MMP-9 was directly involved in the maintenance of a differentiation-resistant phenotype.

In contrast to the induction of MMP-9 expression, RA did not stimulate the expression of TIMP-1, a preferential MMP-9 inhibitor frequently coordinately expressed with MMP-9 and secreted as an MMP-9/TIMP-1 complex, that tightly regulates MMP-9 activation and activity (18). A change in the MMP/TIMP equilibrium is considered a prerequisite for MMP involvement in pathology (47). Our data suggest that within the context of this model of RA differentiation-resistant NB, RA can alter the TIMP/MMP equilibrium by inducing MMP-9 expression without modulating TIMP expression. This adds to other mechanisms responsible for the alteration of MMP/TIMP balance that are of relevance to tumor progression (31, 48, 49).

In addition to NF-κB activation, MMP-9 induction, and alteration of the MMP/TIMP equilibrium, RA also stimulated the capacity of SK-N-BE 9N, but not parental SK-N-BE or SK-N-BE 2N cells, to invade reconstituted BM Matrigel in vitro. MMP-9 involvement in RA stimulated SK-N-BE 9N invasion was confirmed using an anticytotoxic MMP9 antibody (42), which significantly reduced stimulated but not basal levels of invasion. This adds to previous reports that MMP-9 regulates the BM invasivity of tumor cells, including non-N-myc-amplified NB cells in vitro (22, 31, 32, 49) and is relevant to NB behavior in vivo because MMP-9 expression has been linked to aggressive, advanced stage, and metastatic NBs (26, 27). Inhibition of MMP-9 activity, however, did not completely abrogate RA stimulation of SK-N-BE 9N cell invasivity, suggesting additional mechanisms, which may include stimulation of tissue type plasminogen activator and autotaxin expression (detected in SK-N-BE 9N cells; unpublished data), which represent RA regulated genes implicated in NB motility and invasivity in vitro and in vivo (50–52).

In conclusion, although the relationship between N-myc, NF-κB, and resistance to RA-mediated differentiation remains to be clearly defined, our studies indicate that the attainment of an RA differentiation-resistant phenotype by SK-N-BE 9N cells, as a result of altering the relationship between RA and N-myc, can sensitize cells to RA induction of NF-κB activity, subsequent NF-κB-mediated induction of MMP-9 expression that alters the MMP/TIMP equilibrium leading to stimulation of BM invasive behavior in vitro. It follows, therefore, that N-myc-amplified NBs rendered resistant to RA-mediated differentiation by a similar mechanism could exhibit more malignant behavior in the presence of RA in vivo. This not only adds to our understanding of how RA may influence differentiation resistant NBs in vivo but also suggests that inhibitors of NF-κB activity such as the dominant negative IκBα may represent potential inhibitors of this effect.

Materials and Methods

Cells, Media, and Reagents. The human NB cell lines SK-N-BE, SK-N-BE 2N, and SK-N-BE 9N have been described previously (8). Cells were grown in EMEM supplemented with 10% newborn calf serum, antibiotics, and glucose (Euroclone, Wetherby, West York, United Kingdom). All-trans-RA purchased from Sigma (St. Louis, MO) was prepared fresh immediately before use.

Matrigel Invasion Assay. Invasion through reconstituted BM Matrigel (Becton Dickinson, IT) was performed by a modification of a method described previously (53). Briefly, 8 μM Millipore filters were coated with Matrigel (100 μg/filter) at 4°C, and filters were permitted to dry under laminar flow and UV irradiation. Assay filters were reconstituted at 37°C in invasion medium (EMEM 0.1% fraction V BSA) and used to assay for tumor invasion. Cells were grown to confluence, treated for the times indicated with RA, detached by low concentration trypsinization, washed in PBS, and resuspended at a concentration of 0.5 × 10⁶ cells/ml in invasion medium (EMEM/0.1% BSA, with antibiotics, without serum). Cell suspensions were added to the upper well of Invasion chambers and incubated at 37°C for 20 h in the presence or absence of preimmune mouse IgG or monoclonal anticytotoxic MMP-9 antibody (100 μg/ml). Cells that had traversed the filters were counted by light microscopy after staining with hematoxylin and the removal of surface adherent cells.

Preparation of Nuclear Extracts. Nuclear extracts were prepared according to the procedures of Dignam et al., (54) with the following modifications: buffer C contained 20% glycerol; 0.45 M NaCl; and 20 mM HEPES (pH 7.5). Protein inhibitors leupeptin (4 μg/ml) and pepstatin A (4 μg/ml) were added to all buffers. The protein concentrations of extracts
were determined by Bio-Rad assay as outlined by the manufacturer (Bio-Rad, Hercules, CA) and adjusted to 5 μg/ml.

EMSAs. EMSAs were performed as described previously (55). Binding reactions were performed at room temperature for 25 min. The binding reactions contained a 32P 5’-end-labeled, double-stranded oligonucleotide probe, 2 μg of poly(deoxyinosinic-deoxyctydilic acid), 5 μg of nuclear extract, and additional competitor DNAs or antibodies as specified in the figure legends.

The oligonucleotides used in this study were as follows:

- MMP-9AP1
  - 5’-CCCTGACCCCTGAGTCAGCACTTCGGCTGT-3’;
- MMP9mAP1
  - 5’-CCCTGACCCGtcaacGcAGCACTTCGGCTGT-3’;
- MMP-9NF-κB
  - 5’-TGCCCCAGTGGAAATCCCAGCCTTG-3’;
- MMP9-SP1
  - 5’-TGTCCTTCCGCCCCAGATGAA-3’;
- MMP9mSp1
  - 5’-TGTCCTTCCgtcaacCCAGATGAA-3’;
- MMP9Ets
  - 5’-TAGCAGAGCCATTCCTTCCGC-3’;
- MMP9mEts
  - 5’-TAGCAGAGCCgtcaacCCAGC-3’.

All oligonucleotides were double stranded, and the complexing affinity strands are not indicated; lower cases refer to mutated bases.

Antibodies. Antibodies against N-myc, β-actin, cjun, junB, junD, cfos, fosB, fra-1, fra-2, Sp1, Sp2, Sp3, Sp4, p50, p65, and cRel were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anticyclic monoclonal anti-MMP-9 antibody (Calbiochem, CA) and polyclonal anti-MMP9, MMP-2, TIMP-1, and TIMP-2 antibodies have been described previously (30, 42, 56).

Northern Blot Analysis. Total and messenger RNAs were isolated from NB cells, and Northern blots were prepared. Briefly, total RNA was extracted using RNAsol (Iso-Tex, Friendsville, TX) as directed. Polyadenylacidic acid-selected mRNA was purified from total RNA by oligodeoxithymidylate affinity chromatography as described previously (57). Purified mRNA (2 μg) was heat denatured and separated on a 1.4% agarose gel in 10 mM sodium phosphate buffer and transferred to nylon membranes (Hybond N; Amersham). The filters were then exposed to XAR-5 film (Kodak, Rochester, NY). Quantitative loading of RNA was determined by hybridization to a GAPDH control cDNA probe.

Plasmid Constructs. The construct −670MMP9CAT was kindly provided by Drs. Sato and Seiki (University of Kanazawa, Ishikawa, Japan) and contained regulatory sequence of the MMP-9 gene from position 53 to −670 relative to the transcriptional start site (22). The constructs −586MMP9 CAT (NF-κB site deleted), −460MMP9CAT (NF-κB, Sp1, and Ets sites deleted), −88MMP9CAT (NF-κB, Sp1, Ets, and CACA sites deleted), −75MMP9CAT (NF-κB, Sp1, Ets, CACA and AP-1 sites deleted), and −49MMP9CAT (NF-κB, Sp1, ETS,CACA, AP-1, and GT box sites deleted) and the point-mutated constructs −670MMP9mNF-κB (mutated in NF-κB), −670MMP9mSp1 (mutated in Sp1), −670MMP9mAP1 (mutated in AP1), and −670MMP9mGT (mutated in the GT box) have been described previously (31). The heterologous CAT reporter gene constructs bearing a single copy of the MMP-9 NF-κB element (56NF-κBCAT) or a RA response element (tk-TREp2-CAT) have also been described previously (31). The human N-myc expression vector was constructed by inserting 1.6 kb of N-myc cDNA coding sequence into the mammalian expression vector pIRVNeoSV on an EcoRf fragment. Expression vectors for p50 and p65 (44) and the dominant negative mutant IκBα cloned into the mammalian expression vector pmt2T2 (43) were kindly provided by Dr. Ulrich Siebenlist (NIH, Bethesda, MD).

Transient Transfection Assays. Cells were grown on 100-mm Petri dishes. Supercoiled plasmid DNA (7 μg) was transfected into cells using the calcium-phosphate precipitation method (59). All cells were cotransfected with a control plasmid pRSVβgal (1.5 μg; Pharmacia, Uppsala, Sweden) for the estimation of transfection efficiency. After transfection, cells were incubated for an additional 48 h then harvested, and proteins were extracted and prepared for CAT and β-galactosidase assays as previously described (60, 61). The data determined in CAT assay were quantified using a scanning densitometer (Bio-Rad Model GS670) and Molecular Analyst PC software. CAT activity was normalized for variation in transfection efficiency in accordance with data obtained from β-galactosidase control assays.

Immunoblotting. Immunoblots were performed by a modification of a method described previously (62). Briefly, nuclear, total cell extracts or concentrated NB cell culture supernatants were separated by SDS-PAGE under reducing conditions in the presence of precasted broad range molecular weight standards (Bio-Rad). The gels were electroblotted to nitrocellulose membranes (Hybond C+; Amersham) and membranes dried completely before additional processing. Nonspecific protein binding sites were blocked on membranes for 2 h at room temperature using a 5% solution of nonfat dried milk in PBS. Afterward, blocking membranes were washed in PBS and incubated with primary antibody (1–5 μg/ml) diluted in blocking solution for 1–12 h at either room temperature or 4°C for 12-h incubations. The membranes were then washed in PBS and incubated in secondary antibody conjugated to horseradish peroxidase diluted in blocking solution to the recommended concentration depending upon the secondary antibody used. After extensive washing, antigen reactivity was demonstrated by chemiluminescence reaction (Amersham International, Bed ford, United Kingdom). Immunoreactive bands were visualized on Kodak XAR-5 film. Molecular weights were approximated by comparison to precasted molecular weight markers using Molecular Analyst PC software for the Bio-Rad model GS-670 imaging densitometer.

Substrate Gel Electrophoresis. Regular gelatin and reverse zymograms were prepared as described previously (28). Briefly, samples were subjected to regular nonreducing
SDS-PAGE in gels copolymerized with 0.1% gelatin (regular) or 0.1% casein and 30% v/v 72-h serum-free MDA-MB-231 conditioned media (reverse zymogram). After electrophoresis, gels were washed in 2% Triton X-100, rinsed in water, and incubated in a buffer containing 50 mM Tris, 0.2 mM NaCl, and 5 mM CaCl₂ (pH 8.0) overnight (regular) or for 72 h (reverse) at 37°C. Enzyme/substrate activity was assayed by staining with Coomassie Blue.

**RT-PCR for N-myc.** RT-PCR reactions to determine N-myc and GAPDH mRNA expression in transiently transfected SH-EP cells were performed on 1 μg of total RNA using the human N-myc primers 5'-CGACCACAGGGCCCTCAGTA-3' and 5'-CAGGCTTGGTGTTGAGGAG-3' (63) and GAPDH primers 5'-CGGAGTCGGGGAATTCGAGCT-3' and 5'-AGCCCTTCATGTTGAGAGAC-3' (64).

**Statistical Analysis.** The Student’s t test was used for statistical comparison of data. A comparison of means giving t values with associated probabilities of difference < 0.05 was considered to be statistically significant.

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**References**


