Nerve growth factor and retinoic acid inhibit proliferation and invasion in thyroid tumor cells

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Abstract

NGF has anti-proliferative and anti-invasive effects in neuroendocrine tumors. In the present work we examined the effects of NGF and retinoic acid on cell proliferation and invasion in thyroid carcinoma cells. We found that NGF and retinoic acid do not affect cell proliferation on their own but in combination they produce a strong inhibition. We also found that retinoic acid regulates the matrix metalloproteinase 2 activity and invasion. In contrast, NGF inhibited invasion and reverted the effect of retinoic acid. This effect of NGF is likely mediated by an increase in adhesion to laminin and collagen IV and the inhibition of cell migration. NGF also induced the expression of the p75 NGF receptor. In conclusion, NGF and retinoic acid in combination inhibit proliferation and invasion of thyroid papillary carcinoma cells. These data open the possibility of a potential combined therapy for thyroid papillary carcinomas. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Nerve growth factor was initially described as a factor regulating the growth, differentiation and survival of peripheral sympathetic neurons. However, neuroendocrine actions have also been assigned to NGF (Missale et al., 1994, 1995, 1996a; Missale and Spano, 1998). Besides the differentiating action on pituitary cells, we have previously shown that NGF has an anti-tumorigenic activity on neuroendocrine tumors (Missale et al., 1993, 1998; Sigala et al., 1999). NGF inhibits cell proliferation and invasion in pituitary adenomas, small cell lung cancer and prostate cancer cells (Missale et al., 1993, 1998; Sigala et al., 1999). In pituitary adenomas these effects correlate with the expression of the p75 NGF receptor (Missale et al., 1996b). Besides the stimulation of one of its receptors, NGF also stimulates its own production, which establishes an autocrine loop that maintains a non-transformed phenotype in pituitary adenoma cells (Missale et al., 1996b).

Retinoic acid inhibits tumor development at different steps (Lotan, 1996). It has been shown to inhibit cell transformation and proliferation in different models (Bertram, 1983). We have recently shown that retinoic acid also affects late stages of tumor progression. In meningioma cells retinoic acid inhibits invasion through a mechanism that involves an increased adhesion to extracellular matrix components and a consequent inhibition of cell motility (Páez Pereda et al., 1999). Thyroid tumor cells have also been shown to be sensitive to retinoic acid treatment both in vitro and in vivo (Schreck et al., 1994; Schmutzler et al., 1997; Simon et al., 1998). Retinoic acid induces type I 5′-deiodinase activity indicating that it promotes thyroid cell differentiation (Schreck et al., 1994). This mechanism is also effective in patients producing a recovery in iodine uptake in dedifferentiated thyroid tumors (Simon et al.,

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1998). Retinoic acid also induces the intercellular adhesion molecule 1 (ICAM-1) and CD97 protein suggesting that it could modulate cell adhesion and motility (Bassi et al., 1995; Hoang-Vu et al., 1999).

It has previously been shown that retinoic acid can modify the sensitivity to NGF in different models. The mechanism of this interaction involves the differential regulation of NGF receptor expression by retinoic acid. In neuroblastoma cells, for example, retinoic acid modulates NGF responsiveness through the regulation of trkA NGF receptor expression (Matsumoto et al., 1995; Bogenmann et al., 1998) while in sympathetic neurons, retinoic acid inhibits trkA expression (Kobayashi et al., 1998). In pheochromocytoma PC12 cells retinoic acid stimulates p75 NGF receptor subunit expression, thereby making these cells more sensitive to the differentiating effects of NGF (Scheibe and Wagner, 1992). On the other hand, other cytokines have also been shown to potentiate the anti-tumorigenic and differentiating effects of retinoic acid in different cell types (Lancillotti et al., 1995; Kobayashi et al., 1998; Lingen et al., 1998).

In this work we examined the combined effects of NGF and retinoic acid on cell proliferation, in vitro invasion, metalloproteinase activity, migration, adhesion to extracellular matrix components and p75 NGF receptor expression in a thyroid papillary tumor cell line.

2. Materials and methods

2.1. Materials

Unless stated, all reagents were from Sigma (St. Louis, MO, USA), Roche Molecular Biochemicals (Penzberg, Germany) or Pharmacia (Uppsala, Sweden).

2.2. Cell culture

The cell culture reagents and materials were purchased from Gibco BRL Life Technologies (Eggenstein, Germany), Seromed (Berlin, Germany), or Nunc (Wiesbaden, Germany). 8505-C cells were cultured as previously described (Aust et al., 1997; Hofmann et al., 1998; Hoang-Vu et al., 1999) in DMEM-F12 medium with 10% fetal calf serum, 0.1 mmol l⁻¹ glutamine, 2.5 mg l⁻¹ amphotericin-B, and 10³ U l⁻¹ penicillin/streptomycin. For NGF and retinoic acid treatments the culture medium was replaced with stimulation medium (DMEM pH 7.3 containing 2.2 g l⁻¹ NaHCO₃, 10 mmol l⁻¹ HEPES, 2 mmol l⁻¹ glutamine, and 1 g l⁻¹ BSA). Then, 50 ng ml⁻¹ mouse NGF 2.5S (Roche Molecular Biochemicals, Penzberg, Germany) was added every 2 days for a total of 4 days. 10⁻⁵ mol l⁻¹ all-trans-retinoic acid or 100 µU ml⁻¹ bovine TSH (Sigma, St. Louis, MO, USA) was added for the last 24 h. At the end of the treatment, the medium was collected for zymographic analysis and total protein measurements. The cells were collected with citrate buffer and then used for in vitro invasion, migration and adhesion assays previously described (Páez Pereda et al., 1999; Sigala et al., 1999). After treatment with NGF or retinoic acid and before using them in invasion, migration or adhesion assays all the cell preparations were counted using acridine orange–ethidium bromide staining in order to rule out any cytotoxic or apoptotic effects. No cell death was observed after 24 or 96 h treatments.

2.3. Cell proliferation

After 3 days of NGF treatment retinoic acid and 0.1 µCi ml⁻¹ [³H]-thymidine were added to the cultures. After a further 24-h period, the medium was removed and the cells treated with 10% trichloroacetic acid. [³H]-thymidine was measured by liquid scintillation as previously described (Arzt et al., 1993). At the end of the NGF and retinoic acid treatment the cell vitality was measured by acridine orange–ethidium bromide staining. No differences were observed among the different treatments and there was no indication of increased cytotoxicity.

2.4. In vitro invasion

The invasive potential of 8505-C cells was analyzed as we have previously described (Missale et al., 1998; Páez Pereda et al., 1999; Sigala et al., 1999; Páez Pereda et al., 2000). Polycarbonate filters, with an 8-µm pore size, in 24-transwell chambers were covered with 30 µg of Matrigel (Becton Dickinson, Heidelberg, Germany) and dried. One hundred thousand cells were added in DMEM containing 0.1% BSA to the inner section of the transwell. Point five percent fetal calf serum was used as a chemoattractant in the outer chamber. The cell preparations had more than 98% of living cells as assessed by acridine orange–ethidium bromide staining. After a 24-h incubation, the cells in the inner chamber were removed with a cotton swab. The cells attached to the bottom side of the membrane were fixed with 4% formaldehyde, stained with hematoxylin, and counted. Three independent experiments were performed, each condition was measured in triplicates and six separate representative microscope fields were counted in each sample. The results were confirmed routinely by independent observers without previous knowledge of the treatment of the samples.
2.5. Cell migration

The in vitro migration of 8505-C cells was analyzed as previously described (Páez Pereda et al., 1999). Twenty thousand cells were seeded in Boyden chambers on polycarbonate filters covered with 5 $\mu$g cm$^{-2}$ collagen IV or laminin. Point five percent fetal calf serum was used as a chemoattractant in the lower chamber. After a 24-h incubation, the cells in the inner chamber were removed with a cotton swab. The cells attached to the bottom side of the membrane were fixed with 4% formaldehyde, stained with hematoxylin, and counted. Three independent experiments were performed and evaluated as described for the invasion assays.

2.6. Short term cell adhesion

Cell adhesion was measured as previously described (Páez Pereda et al., 1999). Briefly, 10 000 cells per well were seeded into 96 well plates previously coated with 5 $\mu$g cm$^{-2}$ collagen IV or laminin (Becton Dickinson, Heidelberg, Germany). After a 3-h incubation, the plates were shaken for 1 min at 150 rpm and washed with PBS to remove any non-adherent cells. The attached cells were fixed with 4% formaldehyde, stained with hematoxylin, and counted. Three independent experiments were performed and evaluated as described for the invasion assays.

2.7. Zymographic analysis of matrix metalloproteinases (MMPs)

Gelatinolytic activity was analyzed as previously described (Páez Pereda et al., 1999, 2000) using 10% polyacrylamide gels containing 0.2% gelatin. Conditioned media obtained as described earlier were mixed with loading buffer containing 2.5% SDS and incubated for 30 min at room temperature before loading onto the gel. After electrophoresis at 4°C under non-denaturing conditions, the gels were washed as we previously described (Páez Pereda et al., 1999, 2000). After staining with Coomassie blue R250, gelatinase activity was observed as clear zones of proteolysis against a blue background. Incubation of the gels in the presence of 20 mmol l$^{-1}$ EDTA was performed to demonstrate the Ca$^{2+}$ and Zn$^{2+}$ dependence of the proteinase activity observed.

2.8. Polymerase chain reaction

We extracted total RNA as previously described (Arzt et al., 1992; Páez Pereda et al., 1996, 2000) and performed reverse transcription and polymerase chain reaction with specific primers for p75 NGF receptor and actin. The PCR reactions were performed under restrictive conditions using only 20 amplification cycles (1 min, 94°C; 1 min, 55°C; 1 min, 72°C) in order to get band intensities proportional to the amount of RNA present in the samples. Actin was amplified from the same samples under the same conditions as an internal control. The experiments were repeated independently four times. The primer sequences were as follows: p75 NGF receptor 5’GGACAGCCAGAGCTGCA3’, and 5’GGGGATGTGCGCAGTGGAC3’, actin 5’AGCGGGTCAACCACACTGTGC 3’ and 5’CTAGAAGCAT-TGCGTGACCAGATG3’. According to genomic sequences from the genebank, the p75 NGF receptor primer sequences are separated by a 655-bp intron. Therefore, genuine amplification of the p75 NGF receptor cDNA results in a 341-bp product, whereas amplification of genomic DNA would result in a 996-bp product. The actin primers are separated by a 483-bp intron, which would result in a 1183-bp product of genomic DNA amplification. In all the experiments a single band corresponding to the expected size of the cDNA products was observed, ruling out genomic DNA contamination.

2.9. Statistics

Statistics were performed using one way analysis of variance (ANOVA) in combination with the Scheffe’s test. The results are expressed as mean ± S.E.

3. Results

3.1. NGF and retinoic acid inhibit thyroid papillary tumor cell proliferation

To evaluate the effects of NGF on thyroid papillary tumor cells, we studied the 8505-C cell line under both basal conditions and during treatment with NGF and subtherapeutical doses of all-trans-retinoic acid. We measured $^3$H-thymidine incorporation to estimate cell proliferation. One hundred $\mu$U ml$^{-1}$ TSH treatment for 24 h was used to demonstrate that the cells were responsive to stimulatory factors (Fig. 1). We observed that 50 ng ml$^{-1}$ NGF or 10$^{-8}$ M retinoic acid on their own did not produce any change in cell proliferation. However, when both substances were added in combination a significant inhibition of cell proliferation was observed (Fig. 1). Vital staining with acridine orange and ethidium bromide did not show any signs of cytotoxicity. Therefore, the inhibition produced by NGF and retinoic acid is not due to a toxic effect.

3.2. NGF inhibits invasion of thyroid papillary tumor cells

To examine the effects of NGF on the invasive potential of thyroid papillary tumor cells we measured...
the in vitro invasion of 8505-C cells under basal conditions or under NGF and retinoic acid treatment. We observed that $10^{-8}$ M retinoic acid on its own induced a significant increase in invasion. By contrast, 50 ng ml$^{-1}$ NGF produced an inhibitory effect. NGF also reverted the stimulation produced by retinoic acid to basal levels (Fig. 2).

Fig. 1. NGF and retinoic acid regulate thyroid papillary tumor cell proliferation. 8505-C thyroid papillary tumor cells were treated for 4 days with 50 ng ml$^{-1}$ NGF and for 24 h with $10^{-8}$ M all-trans-retinoic acid (RA) and 100 $\mu$U ml$^{-1}$ TSH as indicated. Cell proliferation was measured by $^3$H-thymidine incorporation as described in Section 2. * $P < 0.01$ as compared to the control values. ** $P < 0.01$ as compared to NGF or retinoic acid on their own. These results are representative of three independent experiments.

3.3. NGF inhibits MMP-2 production in thyroid papillary tumor cells

Tumor invasion depends on the degradation of extracellular matrix by matrix metalloproteinases (MMPs) (Stetler-Stevenson et al., 1993; Aust et al., 1997; Hoffman et al., 1998). Therefore, using gelatine zymogram we measured the production of MMP-2 and MMP-9 in 8505-C cells with NGF and retinoic acid treatment. We observed that 8505-C cells under basal conditions secreted proMMP-2, detected as a 72 kDa band, but MMP-9 activity was undetectable (Fig. 3). The identity of proMMP-2 bands was confirmed by co-migration with proMMP-2 from previously characterized melanoma and pituitary cell lines (not shown). We found that retinoic acid stimulates proMMP-2. This stimulation is in agreement with the effect of retinoic acid on in vitro invasion. NGF produced a slight inhibition of MMP-2 activity and reverted the stimulatory effect of retinoic acid (Fig. 3).

3.4. NGF modulates thyroid papillary tumor cell motility

Beside the degradation of the extracellular matrix, invasion depends on the ability of the cells to dynamically bind and migrate on an extracellular matrix substrate. To test the possible effects of NGF and retinoic acid on these mechanisms we measured short-term cell adhesion as an integrative parameter of the affinity of thyroid tumor cells for collagen IV and laminin, the main components of the basement membrane. We found that 50 ng ml$^{-1}$ NGF both on its own and in combination with $10^{-8}$ M retinoic acid stimulated cell adhesion to collagen IV and laminin (Fig. 4). We then evaluated the effects of NGF and retinoic acid on cell motility by measuring cell migration on the same extracellular matrix substrates. We observed that NGF on its own and in combination with retinoic acid inhibited cell migration on collagen IV and laminin (Fig. 5).
Therefore, the increase in cell adhesion to laminin and collagen IV produced by NGF and retinoic acid correlates with the inhibition of cell motility observed in the migration assays. To rule out a possible effect of retinoic acid or NGF on the responsiveness to chemoattractant factors, we performed migration assays on Boyden chambers without extracellular matrix substrates. Under these conditions NGF and retinoic acid did not have any effect compared to untreated cells, indicating that the NGF and retinoic acid effects are not mediated by differences in the responsiveness to chemoattractants (not shown).

3.5. NGF stimulates p75 NGF receptor expression

Since we have previously observed that NGF-induced differentiation of pituitary adenoma cells involves the expression of the p75 NGF receptor we measured p75 expression in thyroid papillary tumor cells by RT-PCR (Missale et al., 1996b). We observed that 8505-C cells did not express the p75 NGF receptor under basal conditions. Reamplification for 30 cycles of the control samples from four independent experiments consistently failed to produce a PCR product confirming the absence of p75 NGF receptor expression in these cells under basal conditions. However, 50 ng ml$^{-1}$ NGF on its own and in combination with $10^{-8}$ M all-trans-retinoic acid (RA) as indicated. At the end of the treatment the cells were collected for migration assays on Boyden chambers covered with laminin (A) or collagen IV (B) as described in Section 2. * $P < 0.01$ as compared to control values. These results are representative of three independent experiments.

4. Discussion

Thyroid papillary tumors constitute 80% of all thyroid tumor types. Although these tumors have in general a good prognosis after surgical treatment, a significant number of cases can evolve malignant phenotypes, losing the ability to incorporate iodine and precluding the application of $^{125}$I therapy (Clark, 1996). The mechanisms controlling this process are not understood and therapeutic tools to control the invasion of these tumors still need to be developed (Farid et al., 1994; Clark, 1996). In the present study we explore the mechanisms of tumor invasion in thyroid papillary tumor cells and describe the effects of NGF and retinoic acid on this process. We demonstrate for the first time that NGF alone and in combination with
retinoic acid inhibit different molecular and cellular parameters of tumor progression in thyroid papillary tumor cells. These effects of NGF are similar to the effects we previously found in other tumor types (Missale et al., 1993, 1998; Sigala et al., 1999). Moreover, the mechanisms that mediate the effects of NGF on thyroid tumor cells very likely involve the induction of the p75 NGF receptor as it does in pituitary adenoma cells (Missale et al., 1996b). The effects of NGF on cell adhesion and migration indicate that NGF controls cell motility. These data support, therefore, the model previously found in other tumor types that the stimulation of cell adhesion to the extracellular matrix reduces cell motility and subsequently results in inhibition of invasion (Páez Pereda et al., 1999). This stimulatory effect of NGF on cell adhesion to the extracellular matrix has also been observed in other cell types and therefore could represent a general mechanism for the anti-tumorigenic effects of NGF (Rossino et al., 1990; Zhang et al., 1993).

It was previously shown that matrix metalloproteinases may play a role in thyroid tumor invasion (Aust et al., 1997; Hofmann et al., 1998). Here we show that the induction of MMP-2 by subpharmacological doses of retinoic acid is probably part of the mechanism of invasion in thyroid papillary cells. Retinoic acid, in the same conditions that stimulate in vitro invasion does not affect cell adhesion or migration. Therefore, in our experimental conditions, MMP-2 stimulation could be enough to promote invasion in these tumors. However, we cannot exclude the role of other metalloproteinases or the adhesion to other substrates not tested in this study. In line with these results it has been shown that retinoic acid regulates the expression of molecules involved in cell adhesion in thyroid cells (Hoang-Vu et al., 1999). Other adhesion molecules can also be induced by retinoic acid to produce anti-tumorigenic effects (Hossein et al., 1989; Hossein and Bertram, 1994). On the other hand, NGF only slightly affects MMP-2 activity on its own but strongly stimulates cell adhesion and subsequently inhibits cell migration, indicating that inhibition of cell motility might be sufficient to inhibit invasion in these cells. This mechanism in which increased adhesion to the extracellular matrix without changes in MMP activity results in reduced cell motility and finally in inhibition of invasion has been previously observed in other tumors (Páez Pereda et al., 1999). Moreover, NGF reverts retinoic acid stimulation of MMP-2 activity. Therefore, the effects of retinoic acid and NGF on MMP-2 activity combined with the effects on cell motility result in reduced invasion.

The anti-tumorigenic effects of NGF and retinoic acid probably combine the differentiating effects of NGF and retinoic acid resulting in a non-invasive and less malignant phenotype. Similar interactions between cytokines and retinoic acid have been shown to enhance their anti-tumorigenic effects both in vivo and in vitro (Lancillotti et al., 1995; Lingen et al., 1998). The molecular mechanisms that mediate the interactions between cytokines or growth factors and retinoic acid remain to be clarified. One possible mechanism of interaction involves the NGF-responsive transcription factor NGFI-B, which can form heterodimers with the 9-cis-retinoic acid receptor RXR and regulate gene transcription (Perlmann and Jansson, 1995). On the other hand, COUP-TF, a transcription factor that mediates the anti-proliferative effects of retinoic acid, can induce the expression of the NGF-responsive transcription factor NGFI-A, providing another putative point of crosstalk between NGF and retinoic acid signaling (Pipaon et al., 1999; Lin et al., 2000). Interestingly, it has recently been shown that activation of the NGF responsive factor NGFI-B is not enough to induce the expression of differentiation markers in thyrocytes, including the sodium iodide symporter (Pichon et al., 1999). This could explain the absence of anti-proliferative effects of NGF alone. However, it was previously shown that retinoic acid stimulates the expression of the iodine transporter (Schmutzler et al., 1997), indicating that retinoic acid has differentiating effects on thyroid tumor cells, which could hypothetically complement NGF effects. This mechanism could potentially be used to favor the incorporation of radioactive iodine as a therapy for these tumors (Simon et al., 1998). The fact that retinoic acid inhibits proliferation at subpharmacological doses in the presence of NGF could indicate that NGF could be used to reduce the doses necessary for therapy.
to achieve therapeutic success in patients. Considering that retinoic acid can have secondary effects when applied in high doses, NGF could contribute to the better management of these side effects.

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