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Nerve Growth Factor mediates its pro-invasive effect in parallel with the release of a soluble E-cadherin fragment from breast cancer MCF-7/AZ cells

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To define better the function of Nerve Growth Factor (NGF) in breast cancer progression, we investigated whether this polypeptide was able to induce breast cancer cell invasion. NGF inhibited aggregation of tumour cells through modulation of the E-cadherin/catenin complex function. In addition, NGF induced the breast cancer cells to invade into Matrigel. We focused our attention on how NGF prevents aggregation, in order to discover the signalling pathway that leads tumour cells to acquire the invasive phenotype. Moreover, studies on the identification of signalling pathways that are responsive for NGF-induced invasion will be basically described.

Keywords: E-cadherin, soluble fragment, invasion, Nerve Growth Factor, breast cancer cells.

Cancer cells are dangerous not so much because they have lost the brakes on their growth, but rather because of their ability to metastasize (Finkel, 1999; Hanahan & Weinberg, 2000). This event greatly reduces treatment options and, when tumour cells succeed in spreading, the outcome is devastating, making cancer a lethal disease and representing one of the great challenges in cancer research. It is now well established that cell adhesion has an essential role in regulating metastasis, and loss of such function is a common feature of invasion (reviewed by Bracke et al. 1996; Cavallaro & Christofori, 2001; Mareel & Leroy, 2003). Among classical cadherins, E-cadherin is a transmembrane protein that mediates specific cell-to-cell adhesion through homotypic interactions (Takeichi, 1991). The progression of a carcinoma *in situ* to an invasive cancer is a prerequisite to cancer metastasis and this transition involves the deregulation of the E-cadherin function. Among the various means by which E-cadherin is deregulated, there is currently a great deal of interest in the role of the cleavage of its extracellular part (Noë et al. 2000; Van Aken et al. 2001; Nawrocki-Raby et al. 2003) and the steric hindrance (Vleminckx et al. 1994; Wesseling et al. 1996; Truant et al. 2003) of adhesion proteins in the progression of cancer. Identifying factors that would affect the invasive and adhesive properties by these ways might open future avenues in cancer treatment by way of targeted therapies.

Nerve Growth Factor (NGF) is the prototype of the neurotrophin family of polypeptides that are essential in the development and survival of certain sympatic and sensory neurons in both the central and peripheral nervous system (Dollé et al. 2003a). Besides their original roles in the nervous system, the implication of these polypeptides and their corresponding receptors TrkA, TrkB, TrkC, and $p75^{NTR}$ in neoplasia has received relatively prominent attention in melanoma, prostatic and breast cancer cells (Descamps et al. 1998, 2001; Marchetti et al. 2003; Shonukan et al. 2003). For instance, in breast cancer cells, previous studies have shown that NGF stimulates growth and survival via two distinct signalling pathways. The mitogenic effect of NGF on breast cancer cells required the tyrosine kinase activity of $p140^{trkA}$ as well as the mitogen-activated protein kinase (MAPK) cascade, but was independent of $p75^{NTR}$. In contrast, the anti-apoptotic effect of NGF as well as the activation of the transcription factor NF-κB, but neither $p140^{trkA}$ nor MAPK was necessary. Additionally, we found that NGF contributes to autocrine tumour cell growth (Dollé et al. 2003b). Although NGF has not yet been described as a pro-invasive factor for breast tumour cells, it does increase the invasive properties of pancreatic cancer cells (Okada et al. 2004). The mechanisms by which growth factors cooperate with cell

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adhesion molecules to modulate the epithelial cell invasive phenotype remain poorly understood and still present an important challenge for cancer research. In the present study we investigated the potential role of NGF as a novel regulatory molecule in tumour invasion with regard to the function of E-cadherin.

Materials and Methods

Materials and cell culture

Recombinant human β-NGF was from R&D Systems Europe (Abingdon, UK). HECD-1 is a murine monoclonal antibody recognizing the extracellular domain of human E-cadherin (Takara, Bio Europe, Gennevilliers, France). MB2 is mouse monoclonal antibody raised against MCF-7/AZ cells, recognizing E-cadherin and with neutralizing effects (Bracke et al. 1993). The horseradish peroxidase-conjugated anti-mouse secondary antibody was from Amersham Pharmacia Biotechnology (Little Chalfont, UK). Human breast adenocarcinoma cells MCF-7/AZ variants were cultured in a 50 : 50 mixture of D-MEM and HAM F12 supplemented with 10% fetal bovine serum, and antibiotics (penicillin, 50 i.u./ml; streptomycin, 50 µg/ml). Cells were seeded at 1×10^6 cells/cm^2. For maintenance purposes, cells were passaged weekly, using Moscona's and 0·02% EDTA–0·05% trypsin solution (Life Technologies, Ghent, Belgium). The medium was changed daily in all culture conditions. Cells were maintained at 37 °C in a humidified 10% CO_2, 90% air atmosphere. Cell viability was checked by trypan blue exclusion.

Matrigel invasion assays

A layer of Matrigel^R (Becton Dickinson and Company, Franklin Lakes NJ, USA) (100 µg/cm^2) was prepared in Transwell^R chambers with polycarbonate membrane filters (8 µm pores size) at 37 °C for 2 h. Cells (4×10^5) were seeded on the upper compartment of Transwell^R in serum-containing medium in the presence or absence of NGF at different concentrations (50–300 ng/ml). Conditioned medium from MRC-5 fibroblasts (CM-MRC$_5$) filled the lower compartment and was used as chemoattractant. The invasion assay was performed at 37 °C under a 5% CO_2, 95% air atmosphere. After 48 h, medium and Matrigel were aspirated, and cells on the upper side of the membrane filters were removed with a wet cotton swab. Cells that had invaded the membrane's under surface were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma, St Louis MO, USA) in TBS (Tris Buffer Saline) for 15 min and 20 fields/filter were counted (Hall & Brooks, 2001).

Slow and fast aggregation assays

For slow and fast aggregation assays, human E-cadherin specificity of the aggregation was demonstrated by a treatment using a functionally blocking monoclonal antibody against E-cadherin (MB-2) and was used as described earlier (Bracke et al. 1993; Boterberg et al. 2000). For slow aggregation, single-cell suspensions were seeded on top of a semi-solid agar medium with or without MB-2 and in the presence or absence of NGF at various concentrations (50–300 ng/ml). After 24 h, aggregate formation was evaluated with an inverted phase-contrast microscope at a magnification of ×40. Viability of cells was checked by trypan blue exclusion. For fast aggregation, single-cell suspensions were prepared using an E-cadherin-saving procedure (Kadmon et al. 1990) by using collagenase A treatment followed by trypsin, both in the presence of 0·04 mM-Ca^{2+}. Cells were incubated in an isotonic buffer containing 1·25 mM-Ca^{2+} and DNase, and incubated in BSA-coated wells for 30 min under Gyrotory shaking (New Brunswick Scientific, New Brunswick NJ, USA) at 80 rounds per min for times indicated and at 35 °C. Cells were fixed in 1% glutaraldehyde and the particle diameters were measured in a particle size counter (LS 200; Beckman Coulter, Miami FL, USA) at the start (t_0) and after a 30-min incubation (t_{30}). The relative volume as a function of the particle size was used as an index of aggregation (Noë et al. 1999).

Detection of soluble factors

MCF-7/AZ cells were seeded into 75 cm^2 culture plastic vessels in DMEM/HAM, FCS 10%. Upon confluence, the cells were washed three times with serum-free DMEM and after 24 h the media were replaced with fresh serum-free media and maintained for different periods of time, as indicated, in the presence or absence of NGF at 100 ng/ml. The media were harvested, clarified by centrifugation for 5 min at 1250 ***g*** to eliminate possible cell particles and concentrated by centrifugation through microcentrifuge tubes (Amicon, Millipore, Bedford MA, USA), as per manufacturer's instruction. All samples were denatured under reducing conditions and loaded onto gels for Western blot analysis to observe the release of soluble E-cadherin.

RT-PCR and Western blotting assays

Cells grown to 70% confluence, were washed twice with cold PBS and total cellular RNA was isolated from the breast cancer cells using Rneasy Mini Kit (Qiagen, Leusden, The Netherlands) according to the standard protocols provided in the kit. The amount of extracted RNA was quantified by measuring the absorbance at 260 nm, and the purity of the RNA was checked by the ratio of the absorbances at 260 and 280 nm. The absence of degradation of the RNA was confirmed by electrophoresis of the RNA on a 1·5% agarose gel containing ethidium bromide. RT-PCR analysis was performed for the amplification of E-cadherin and β2-macroglobulin, which was amplified as internal control. RNA (1 µg) was reverse-transcribed using Omniscript^R Reverse Transcriptase for first-strand cDNA synthesis

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(Qiagen). All the reaction mixtures were incubated at 37 °C for 60 min, and at 95 °C for 5 min. After cDNA were generated, PCR was carried out using Taq PCR master mix kit (Qiagen) and the following primers; E-CAD-S (5′GAGAAACAGGATGGCTGAAGG) and E-CAD-AS (5′TGAGGATGGTGTAAGCGATGG) for human E-cadherin that amplified a 300 bp product and β2-MG-S (5′CATCCAGCGTACTCCAAAGA) and β2-MG-AS (5′GA-CAAGTCTGAATGCTCCAC) for β2-microglobulin that amplified a 100 bp product. Amplification of cDNA samples were carried out at the following conditions: 94 °C for 3 min; 35 cycles at 94 °C for 50 s, 50 °C for 50 s and 72 °C for 60 s; followed by 72 °C for 10 min. PCR products, including a DNA ladder (New England Biolabs, Beverly MA, USA) were separated on 2% agarose gel. PCR products were then visualized using ethidium bromide and a u.v. transilluminator.

To prepare protein lysates, the following buffer was added at the time of lysis: PBS, 1% Triton X100, 1% NP-40, phenylmethylsulphonyl fluoride (PMSF), NaF, leupeptin, aprotinin, $NaVO_3$, $Na_4P_2O_7$. Cells were allowed to lyse for 30 min on ice, harvested by scraping in lysis buffer and centrifuged. The supernatants were collected and after boiling for 5 min, all proteins were quantified using BioRad DC Protein Assay (BioRad, Hercules CA, USA). Afterwards, amounts of total proteins of lysates were separated by SDS-PAGE gels, and were transferred onto a nitrocellulose membrane by electroblotting and then incubated with blocking buffer for 1 h. Membranes were then probed with primary antibodies at 4 °C overnight under agitation in blocking buffer, and subsequently labelled using HRP-conjugated secondary antibodies for 1 h. Protein bands were then visualized using enhanced chemiluminescence detection systems with hyperfilm (Amersham Pharmacia Biotechnology, Little Chalfont, UK). In all cases, gels and immunoblot membranes were stained with Coomassie Blue and Ponceau Red to ensure even protein loading and protein transfer, respectively.

Immunofluorescence microscopy

Cells were grown on glass coverslips coated with collagen type 1. Briefly, after washing in ice-cold PBS, cells were fixed with 4% paraformaldehyde for 20 min. Fixed cells were rehydrated with TBS, and then permeabilized in 0·2% Triton X-100/PBS. After fixation and permeabilization, the coverslips were incubated in blocking buffer (5% of bovine serum albumin in TBS) for 1 h. Primary antibodies were diluted in blocking buffer and applied for 1 h. Afterwards, coverslips were washed and incubated with the secondary antibodies for 1 h. Biotinylated antibodies were incubated with Streptavidin/Texas Red in TBS for 30 min. Nuclei were counterstained by DAPI. All incubations were at room temperature. The coverslips were mounted with Glycergel (DAKO, Denmark) and for double labelling, FITC-conjugated anti-E-cadherin and Texas Red-conjugated anti-β-catenin were used. Immunostained cells were visualized using fluorescence microscopy (Dialux20, Leika).

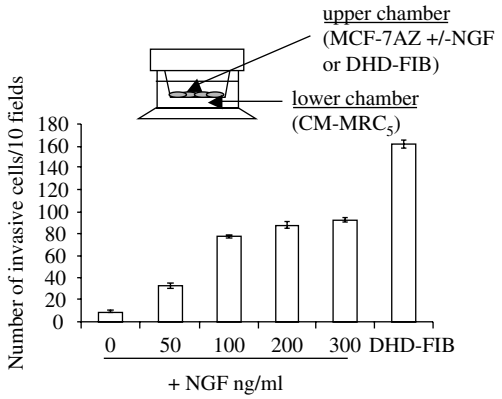

Fig. 1. NGF promotes invasion of MCF-7/AZ cells in Matrigel assay. Malignant cells were seeded on top of the Matrigel and incubated at 37 °C for 24 h in the presence or absence of NGF at various concentrations (50–300 ng/ml). Results are expressed as the number of invasive cells that cross the Matrigel layer. DHD-FIB cells were used as a positive control for invasion. Conditioned medium from MRC-5 fibroblasts (CM-MRC_5) filled the lower compartment and was used as chemoattractant (Scatter Factor/ Hepatocyte Growth Factor).

Statistics

All experiments were performed at least in triplicate. All values are provided as means with SD. Kolmogorov-Smirnov statistics were used to analyse the differences between the cumulative distribution curves obtained in the fast aggregation assay.

Results

To investigate whether NGF affected the invasive and adhesive properties of the breast cancer cells, we used two assays *in vitro* under NGF treatment. Normally, MCF-7/AZ are noninvasive and readily form aggregates *in vitro* (Bracke et al. 1991). First of all, the invasive properties of MCF-7/AZ were assessed in Matrigel assays, as briefly described in Fig. 1. Results were quantified and expressed as numbers of invasive cells that succeed in crossing the Matrigel-coated filter. In the untreated situation, MCF-7/AZ was non-invasive, whereas under NGF stimulation, these cells were invasive (Fig. 1). This invasive effect was dose-dependent. Effects of NGF on cell-cell aggregation have been analysed using both a slow (24–48 h; Fig. 2, left panel) assay under static conditions and fast (30 min) assay under Gyrotory shaking (Fig. 2, right panel). The involvement of E-cadherin in aggregation was further examined by performing the assays in the presence of the anti-E-cadherin antibody MB2. After slow aggregation of single-cell suspensions for 24 h on semi-solid agar, images

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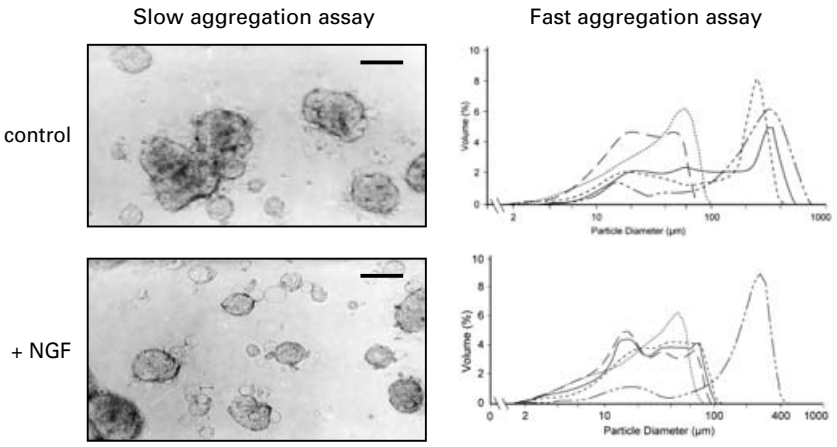

Fig. 2. NGF inhibits cell-cell aggregation of MCF-7/AZ cells. After 24 h on soft agar, in the slow aggregation assay (left panel), aggregate formation and compaction were evaluated with an inverted microscope and pictures of representative cultures were taken. In the absence of NGF (control situation), large compact aggregates of cells were formed, whereas with NGF at 100 ng/ml, these aggregates were smaller. The right panel represents the fast aggregation assay. The volume distribution (ordinate) as a function of particle size (abscissa) was used as an index of aggregation: cell aggregation was measured at the start (t_0: $-MB_2$: dotted thin line) and after a 10 min (t_{10}: point+ line), 20 min (t_{20}: dotted bolt) and 30 min (t_{30}: solid line) aggregation in the absence (upper panel) or presence of NGF at 100 ng/ml (lower panel) after 10 min (t_{10}: point+ line), 20 min (t_{20}: dotted bolt) and 30 min (t_{30}: solid line). Aggregation assays were done at 30 min with antibody against E-cadherin (MB_2), as indicated (dotted big bolt). The impact on aggregation was seen as a shift of the aggregate volume distribution curve to the left. All scale bars = 100 µm.

of representative cultures were photographed. Results showed that MCF-7/AZ in the untreated situation were able to aggregate strongly. In contrast, NGF in the medium reduced the size of aggregates (Fig. 2, left panel). In the fast aggregation assay, after shaking of suspended cell cultures for the indicated times, the results of cell aggregation were similar to those obtained in the previous assays (Fig. 2, right panel).

Disturbances in the expression, distribution or structure of E-cadherin-catenin complex during tumour progression have been described, which perturb cell adhesive function, and lead to the acquisition of a motile and invasive phenotype by cancer cells. So the first step of our work was to study the NGF effect on expression of E-cadherin in MCF-7/AZ cells by RT-PCR (Fig. 3A) and Western blotting (Fig. 3B). Total protein extracts from MCF-7/AZ cells treated with NGF showed neither modification of the levels of E-cadherin protein as compared with untreated cells (Fig. 3B), nor of its mRNA levels (Fig. 3A). In addition, in cell fractionation experiments, the relative amounts of E-cadherin and β-catenin in detergent-extractable and detergent-non-extractable fractions were not affected with NGF (results not shown). These experiments converged in the same way: NGF acts on invasion without any effect on expression of E-cadherin. The correct function of E-cadherin/catenins complex requires its association with the cytoskeleton. As alterations of such complex have been implicated in the acquisition of the invasive phenotype by cancer cells, we analysed whether NGF modulates its subcellular distribution. For this reason, we tested the impact of NGF on the distribution of E-cadherin and β-catenin within the cell by immunocytochemistry. MCF-7/AZ cells form an epithelioid monolayer on tissue culture plastic substratum, indicating that under those circumstances, the E-cadherin/catenin complex is active. The results showed that NGF had no impact on the cellular distribution of E-cadherin and β-catenin (Fig. 4). Thus, the acquisition of an invasive phenotype did not result from a difference in the level or distribution of E-cadherin, but from another mechanism affecting the function of this molecule.

Since the release of the E-cadherin ectodomain (sE-CAD) (M(r) 80 000) has already been described as an invasion promoter in breast tumour cells, we suspected that NGF might act on E-cadherin shedding. In this study, sE-CAD levels were measured with Western blotting analysis and the results showed that the E-cadherin ectodomain was constitutively shed from the surface of MCF-7/AZ cells after 24 h of culture (Fig. 5). In contrast, in the NGF situation, the condition medium contained sE-CAD after 10 min of NGF stimulation, whereas no detection has been observed in the untreated situation at 10 min. So NGF increased the level of sE-CAD as detected in conditioned media, but did not decrease the level of full-length (M(r) 120 000) E-cadherin (Fig. 3B). Further studies are needed to determine whether NGF induces the release of the extracellular domain of E-cadherin by an enzymic cleavage, resulting in its dissociation from the cadherin/catenin complex or whether sE-CAD, possibly pre-existing in cytoplasmic vesicles, is secreted under NGF stimulation.

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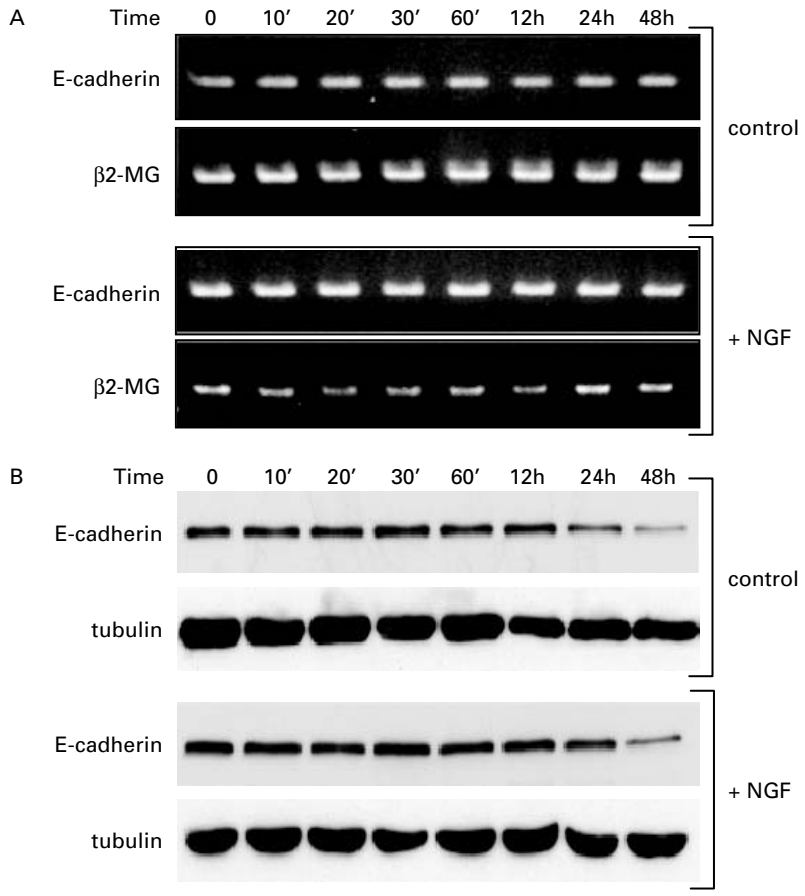

Fig. 3. Effect of NGF on expression of E-cadherin in MCF-7/AZ cells. NGF was added to the media at different time points (from 0 min to 48 h) and the expression of E-cadherin was analysed by RT-PCR and Western blot. A. RT-PCR analysis of E-cadherin mRNA levels in untreated cells (upper panel) and NGF stimulated cells (lower panel) for different periods of time. β2-MG (β2-microglobulin was used as an internal control. The expected sizes of RT-PCR products of E-cadherin and β2-MG were 300 bp and 100 bp respectively. B. Western blotting of total protein extracts of MCF-7/AZ. Staining was done with antibodies HECD-1 against E-cadherin (120 kDa) and tubulin (50 kDa). Tubulin was used as an internal control. No bands were seen when the first antibody was omitted.

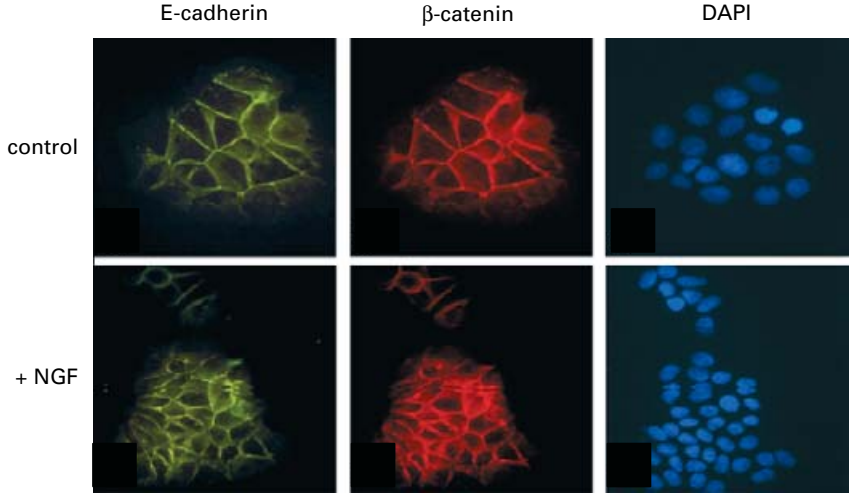

Fig. 4. Effect of NGF on distribution of the E-cadherin/catenin complex in MCF-7/AZ cells. Immunofluorescence of the E-cadherin/catenin complex was used to observe the cellular disbribution of E-cadherin and β-cantenin by fluorescence microscopy in MCF-7/AZ in the presence or absence of NGF 100 ng/ml. Green FITC and Texas Red fluorescence were used for E-cadherin and β-catenin respectively. DAPI was used to indicate the nuclei of tumour cells. Scale bar: μm.

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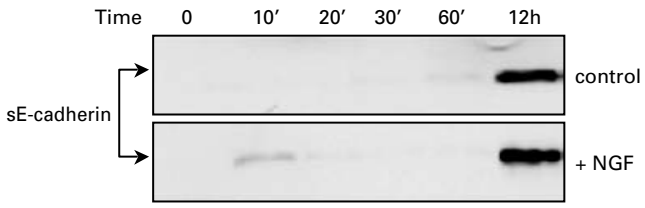

Fig. 5. Effect of NGF on release of soluble E-cadherin fragments from MCF-7/AZ. Cells were incubated in serum-free medium for different periods of time (0 min to 12 h) in the presence (lower panel) or absence (upper panel) of NGF at 100 ng/ml. The media were harvested, concentrated and sE-CAD fragments (80 kDa) were analysed by SDS-PAGE, blotted and immunostained with HECD-1.

Discussion

Tumourization of mammary epithelium is under the influence of growth factors, which can stimulate or inhibit proliferation, migration, survival and invasion of breast cells (Ethier, 1995). The emergence of cells with increased sensitivity for growth factors is now considered an important mechanism leading to carcinogenesis (Hanahan & Weinberg, 2000), and interactions with cadherins play essential roles in the development of cancer as well (Comoglio et al. 2003). Our study showed a new biological effect of NGF on breast cancer cells, resulting in an induction of invasion, as demonstrated by assays *in vitro*. Moreover, the present results indicate that the downregulating mechanism of E-cadherin function induced by NGF might consist in remodelling of the full length of E-cadherin. Reduced expression of E-cadherin, frequently observed under stimulation of some pro-invasive factors, has not been implicated in our model. Boterberg et al. (2000) described the internalization of the E-cadherin/catenin complex of MCF-7/AZ cells after treatment with conditioned medium from skin squamous carcinoma cells as pointing towards the downregulating mechanism for this complex. With biotinylation of cell surface protein experiments we showed that NGF did not induce the internalization of E-cadherin and so could not explain the loss of cell adhesion and the acquisition of the invasive phenotype of MCF-7/AZ cells (results not shown). Furthermore, preliminary results (not shown) showed that NGF-mediated invasion required the induction of TrkA and MAP-kinase activities as well as the requirement of $p75^{NTR}$, whereas mitogen and survival NGF effects were mediated through two distinct signalling pathways. Indeed, the mitogenic effect of NGF on breast cancer cells required the tyrosine kinase activity of $p140^{trkA}$ as well as the mitogen-activated protein kinase (MAPK) cascade, but was independent of $p75^{NTR}$. In contrast, the anti-apoptotic effect of NGF as well as the activation of the transcription factor NF-κB was needed, but neither $p140^{trkA}$ nor MAPK was necessary (Descamps et al. 1998, 2001). In parallel with previous studies that show NGF to be overexpressed by breast cancer cells, resulting in an autocrine stimulation of tumour cell growth (Dollé et al. 2003b), the present work indicated an autocrine stimulation of tumour invasion by NGF through the release of E-cadherin fragments. Thus, taken together, the results show NGF and its receptors as well as their signal to look increasingly promising as potential drug targets for breast cancer (Dollé et al. 2004).

Disruption of E-cadherin function is considered a prominent step in progression towards the invasive phase of carcinoma. Even though several mechanisms have already been described at the genomic and post-translational levels, it is becoming clear that other occurrences are not yet known or remain elusive. In fact, the mechanism for inhibition of E-cadherin-mediated cell-cell adhesion by proteolytic shedding of the ectodomain is well illustrated (Noë et al. 2000). The study of the effect of still other factors that can modulate the function of the E-cadherin function in MCF-7/AZ cells and their mechanism of action will be the subject of our future work. Indeed, we will investigate whether NGF cleaves the full length E-cadherin molecule by proteolysis or whether NGF releases pre-existing sE-CAD fragments contained in cytoplasmic vesicles.

In summary, the results of the present study support a role for NGF in the regulation of the invasive phenotype of MCF-7/AZ breast tumour cells. However, more information is needed concerning the suggested shedding of E-cadherin protein. Further studies on how NGF gives rise to release of sE-CAD are needed to elucidate the mechanism of action of this growth factor on invasion.

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