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Overexpression of the *RON* gene in human breast carcinoma

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Constitutive activation of the *RON* gene, known to code for the tyrosine-kinase receptor for Macrophage Stimulating Protein (also known as Scatter Factor 2), has been shown to induce invasive-metastatic phenotype *in vitro*. As yet, nothing is known about the expression of this novel member of the *MET*-oncogene family in spontaneously occurring human cancers. Here we report that *Ron* is expressed at abnormally high levels in about 50% primary breast carcinomas (35/74 patients). Among these, the expression is increased more than 20-fold in 12 cases and the overexpressed protein is constitutively phosphorylated on tyrosine residues. Notably, *Ron* is only barely detectable in epithelial cells of the mammary gland, and its expression remains unchanged in benign breast lesions (including adenomas and papillomas). Overexpression was observed in different histotypic variants of carcinomas; it is associated with the disease at any stage and correlates with the post-menopausal status. In breast carcinoma cells grown *in vitro*, activation of the *Ron* receptor resulted in proliferation, migration and invasion through reconstituted basement membranes. Altogether, these data suggest a role for the *RON* gene in progression of human breast carcinomas to the invasive-metastatic phenotype.

Keywords: protein-tyrosine kinase; *RON*; breast neoblasts

Introduction

The *RON* gene encodes a transmembrane tyrosine kinase which has been identified as the receptor for MSP/SF2 (Macrophage Stimulating Protein/Scatter Factor-2, also known as Hepatocyte Growth Factor-Like, HGF-L) (Gaudino *et al*., 1994; Wang *et al*., 1994). In epithelial cells, Scatter Factors induce a distinctive biological response that involves cytoskeletal reorganization, loss of intercellular junctions and cell-dissociation, followed by active migration (Gherardi *et al*., 1989). Cell 'scattering' is associated with invasion of extracellular matrices (Stoker *et al*., 1987; Weidner *et al*., 1990; Giordano *et al*., 1993).

RON belongs to the *MET* oncogene family (Gaudino *et al*., 1994): *MET*, the prototype of the family, codes for the receptor for HGF/Scatter Factor-1 (Naldini *et al*., 1991); *SEA*, another member of the family, encodes an orphan tyrosine kinase receptor (Huff *et al*., 1993). The strong homology between the members of this family is based on structural and biochemical features: (a) the α-β-heterodimeric structure (Giordano *et al*., 1989; Gaudino *et al*., 1994); (b) two neighboring phospho-tyrosine residues in the kinase domain responsible for up-regulation of the catalytic activity (Ferracini *et al*., 1991); (c) a two-tyrosine multifunctional docking site in the C-terminal tail endowed with the uncommon property of concomitantly activating multiple SH2-containing intracellular signal transducers (Ponzetto *et al*., 1994). In the *Met* receptor, phosphorylation of these two tyrosines triggers the intracellular signaling cascade eliciting the distinctive biological response leading to growth, 'scattering', morphogenesis in physiological conditions (Montesano *et al*., 1991; Medico *et al*., 1996) and to invasive growth in cancer cells *in vitro* (Giordano *et al*., 1993; Rong *et al*., 1994). Recently, missense mutations located in the tyrosine kinase domain of the *MET* gene have been identified in the germline of affected members of hereditary papillary renal carcinoma families and in a subset of patients affected by sporadic renal carcinomas of the same histotype. These are likely to be gain-of-function mutations which lead to constitutive activation of the *Met* protein and to tumorigenesis in renal tissue (Schmidt *et al*., 1997).

The *RON* gene is expressed in a number of epithelial cells, in lineages of hematopoietic origin (such as granulocytes and monocytes) in cells of neuro-ectodermal origin and in osteoclasts (Gaudino *et al*., 1994, 1995; Kurihara *et al*., 1996). In liver progenitor cells grown *in vitro*, *Ron* activation results in 'scattering', invasion of the extracellular matrix and formation of tubular duct-like structures (Medico *et al*., 1996).

So far, nothing is known about a possible oncogenic potential of the *RON* gene in human pathology. Constitutive activation of the *Ron* kinase (obtained by replacement of the *Ron* extracellular domain with a protein dimerization motif) induces a motile-invasive phenotype, characterized by constitutive cell dissociation, 'scattering', invasion of extracellular matrices and formation of aberrant tubular structures *in vitro* (Santoro *et al*., 1996). Moreover, a naturally occurring *Ron* protein variant, constitutively activated in a gastric carcinoma line, was found to be responsible for the acquisition of invasive properties *in vitro*. This variant is an uncleaved single-chain protein which undergoes disulfide-linked intracellular dimerization (Collesi *et al*., 1996).

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At present, no information is available on a possible role of *RON* in spontaneously occurring human cancers. In this study we focus on human breast carcinomas, as the biological responses controlled by the *RON* gene namely growth, invasion and induction of tubular structures, may very well explain some biological features of these tumors. We found that the *Ron* receptor is overexpressed in a significant proportion of carcinomas and that its activation *in vitro* mediates proliferation, motility and matrix invasion of breast cancer cells.

Results

Expression of the Ron *receptor in normal mammary gland, benign lesions and malignant breast tumors*

A total of 94 breast tissue specimens of the following categories were examined for the *Ron* receptor expression: (a) normal mammary glands ($n=8$); (b) benign lesions ($n=12$); (c) primary ductal or lobular carcinomas ($n=74$). The *Ron* protein (p150β^{RON}) was barely detectable in the normal mammary gland tissue and in benign lesions including fibroadenomas, papillomas and tissues with microcalcifications (Figure 1a and b). Interestingly, in primary breast carcinomas the *Ron* receptor was expressed at higher levels than in benign lesions in 35 out of 74 cases, accounting for 47% of the total (Figure 1c). In the majority of these tumors (23 cases, 31% of the total) the level of expression was increased from 2–20-fold as compared to the amount of protein detectable in benign epithelium. In 12 cases (16% of the patients examined) carcinoma cells overexpressed the *Ron* protein with an impressive increase of more than 20-fold. A single patient showed a breast carcinoma overexpressing *Ron* more than 70-fold (Figure 1c, case 74), determined by densitometric analysis of serial dilution of samples in Western blots (data not shown). To exclude the possibility that different amounts of *Ron* protein merely reflected the relative number of epithelial cells in the samples, these data were normalized to the content of K19 cytokeratin marker.

Immunohistochemical analyses performed on selected paraffin-embedded tumor specimens demonstrated that the *Ron* receptor is localized on tumor cells. Figure 2 illustrates representative results and shows intense staining of epithelial both in ductal and lobular carcinomas. In all cases the stroma and endothelial cells were not stained.

To investigate the possibility that elevated expression of p150β^{RON} leads to its enzymatic activation in human breast carcinoma, we analysed phosphotyrosine-containing proteins in primary tumors in relationship to *Ron* expression. As shown in Figure 3, we found a strong correlation between elevated expression of p150β^{RON} and its phosphorylation.

To investigate whether the overexpression of *Ron* receptor observed in breast carcinomas was due to amplification or to genomic rearrangements, Southern blot analysis was performed. The resulting restriction pattern and hybridization intensities gave no evidence of rearrangements or amplification (data not shown).

The primary breast carcinomas examined were further classified according to their histological and clinical features (see Materials and methods). Clinicopathological parameters were related to the level of *Ron* receptor expression. *Ron* overexpressors belonged to different histotypic variants. The relatively small number of cases (74 primary carcinomas) did not allow to establish significant links between *Ron* overexpression and estrogen or progesterone receptors, *erb*B2 and *p53* expression (data not shown). However, a significant correlation was found between *Ron* overexpression and the post-menopausal status of the patients (Figure 4).

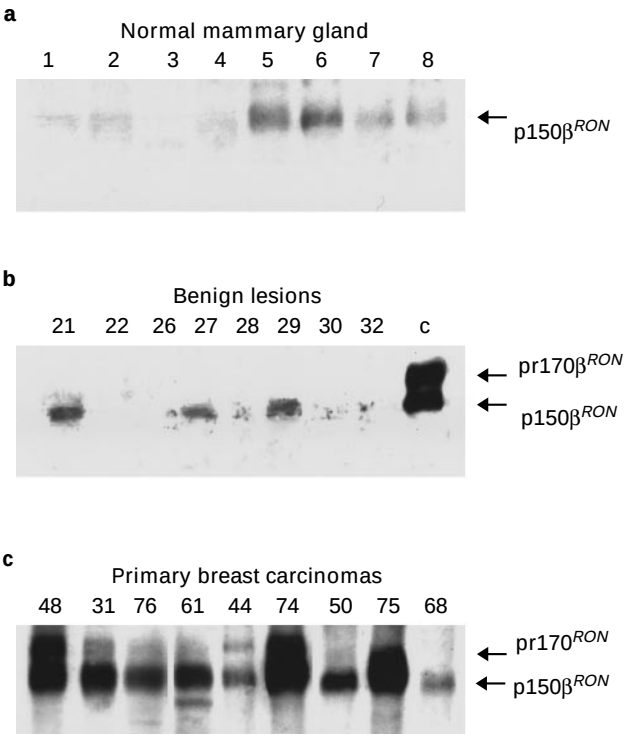

Figure 1 Expression of the *Ron* receptor in normal tissues (**a**), benign lesions (**b**) and in primary breast carcinomas (**c**). The amount of detergent-solubilized proteins loaded in each lane was previously normalized to the content of the epithelial marker k19 cytokeratin. Proteins were immunoprecipitated with a polyclonal antibody raised against the human *Ron* receptor, separated under reducing conditions, transferred to nitrocellulose paper and stained with the same antibody. The arrows point to the 150 KDa β chain of *Ron* (p150β^{RON}). In cells expressing high levels of *Ron* the 170 KDa precursor (pr170^{RON}) was also evident as a tightly compressed double band. Figures show representative cases from the total samples examined. Numbers on the top of each lane indicate different patients. Proteins extracted from the GTL16 gastric cancer cell line were used as positive control (**c**). In (**b**), cases no. 21 and 22 were tissues with microcalcifications; no. 26 and 27 were papillomas; no. 28, 29, 30 and 32 were fibroadenomas. In **c**, cases no. 48, 74 and 75 expressed amounts of the *Ron* receptor over 20-fold higher than the mean of proteins detectable in benign lesions. Case no. 74 overexpressed *Ron* more than 70-fold

Activation of the Ron *receptor induces an invasive phenotype in breast cancer cells* in vitro

Expression of the *Ron* receptor was examined in different human breast cancer cell lines. The receptor was transcribed and expressed in all the five tumorigenic cell lines analysed (data not shown). ZR75.1 cells – showing levels of *Ron* expression comparable to levels found in human tumor samples

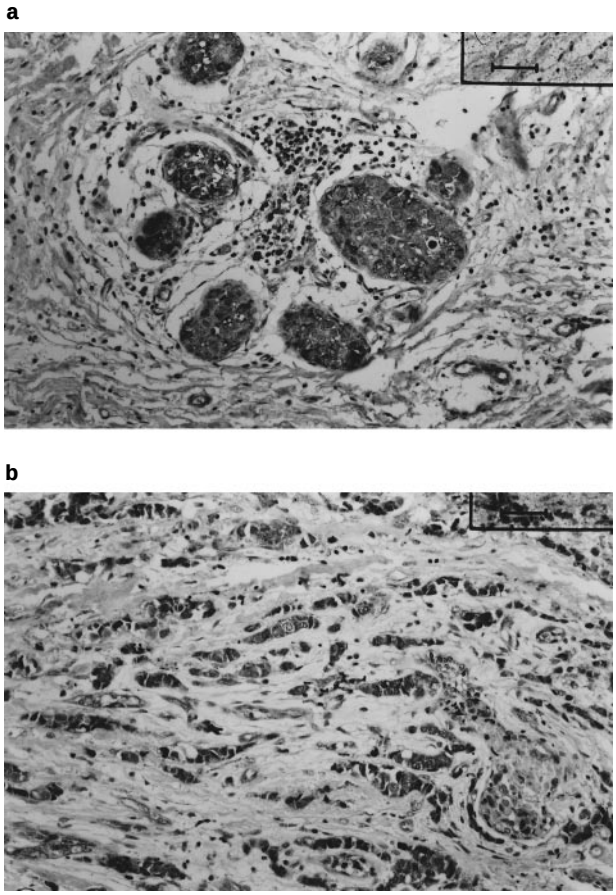

Figure 2 Immunohistochemical staining for the *Ron* receptor in human breast tumors. Breast carcinoma specimens were stained using polyclonal antibody raised against the human *Ron* receptor. Positivity is displayed only by neoplastic epithelial cells both in ductal (**a**) and lobular tumors (**b**). Note the predominantly membrane distribution of the stain. The surrounding stroma and the endothelial cells are negative. Space bar corresponds to 50 μm

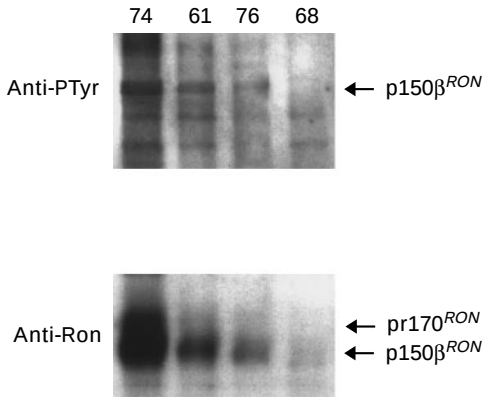

Figure 3 Tyrosine phosphorylation of the *Ron* receptor in breast tumors. Proteins from primary breast tumor were lysed, separated under reducing conditions and immunoprecipitated with the anti-*Ron* antibody. Western blot was stained both with anti-phosphotyrosine and anti-*Ron* antibodies. The *Ron* receptor phosphorylation is increased in overexpressing tumors (cases no. 74, 61)

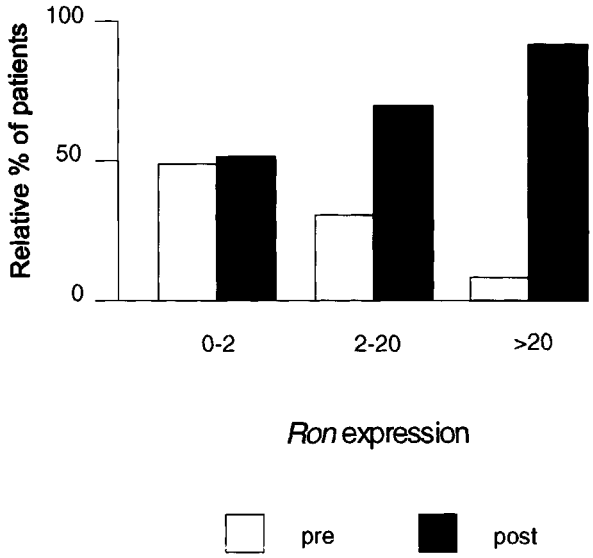

Figure 4 Correlation between the *Ron* receptor expression and menopausal status ($P<0.04$). Levels of the *Ron* receptor expression were evaluated by laser densitometric analysis of X-ray films. Relative scores are: 0–2 = comparable expression to the mean value of benign lesions (39 cases out of 74); 2–20 = from 2- to 20-fold increase of the mean value of benign lesions (23 cases out of 74); >20 = more than 20-fold increase (12 cases out of 74). Statistical significance was assessed by *P* value for the χ^2 test or Fisher exact test

– were used as model to study the biochemical and biological responses of cancer cells to MSP/SF2. In these cells the kinase was functionally active, as MSP/SF2 stimulation triggered tyrosine phosphorylation (Figure 5a). Nanomolar concentrations of MSP/SF2 induced proliferation after 4 h (Figure 5b). The motogenic response was studied in a chemotactic assay by seeding cells in the upper chamber of a Transwell plate in which the ligand was added to the lower chamber, as previously described (Santoro *et al*., 1996). MSP/SF2 was found to stimulate cell migration and behaves as a strong chemo-attractant (Figure 5c). The mitogenic and motogenic responses were simultaneously evaluated in a wound healing assay, which tests the ability of filling artificial gaps created in cell monolayers (Bussolino *et al*., 1992). ZR75.1 cells responded to MSP/SF2 and completely repaired the wound in 72 h (Figure 5d). The invasive-metastatic phenotype was assessed *in vitro* by a chemoinvasion assay performed using Transwell chambers coated with a reconstituted basement membrane containing collagen IV, laminin and glycosaminoglycans (Kobayashi *et al*., 1992). As shown in Figure 5d, ZR75.1 cells invaded and crossed the basement membrane migrating towards the ligand gradient determined by the presence of MSP/SF2 in the lower compartment.

Discussion

Breast cancer is a slow-growing tumor characterized by a long pre-clinical period. Cancerous cells from intraductal lesions suddenly acquire the capacity to invade the surrounding stroma. The molecular mechanism underlying the acquisition of the invasive phenotype is still largely unknown. Genetic alterations that accumulate in breast cancer, including those of the

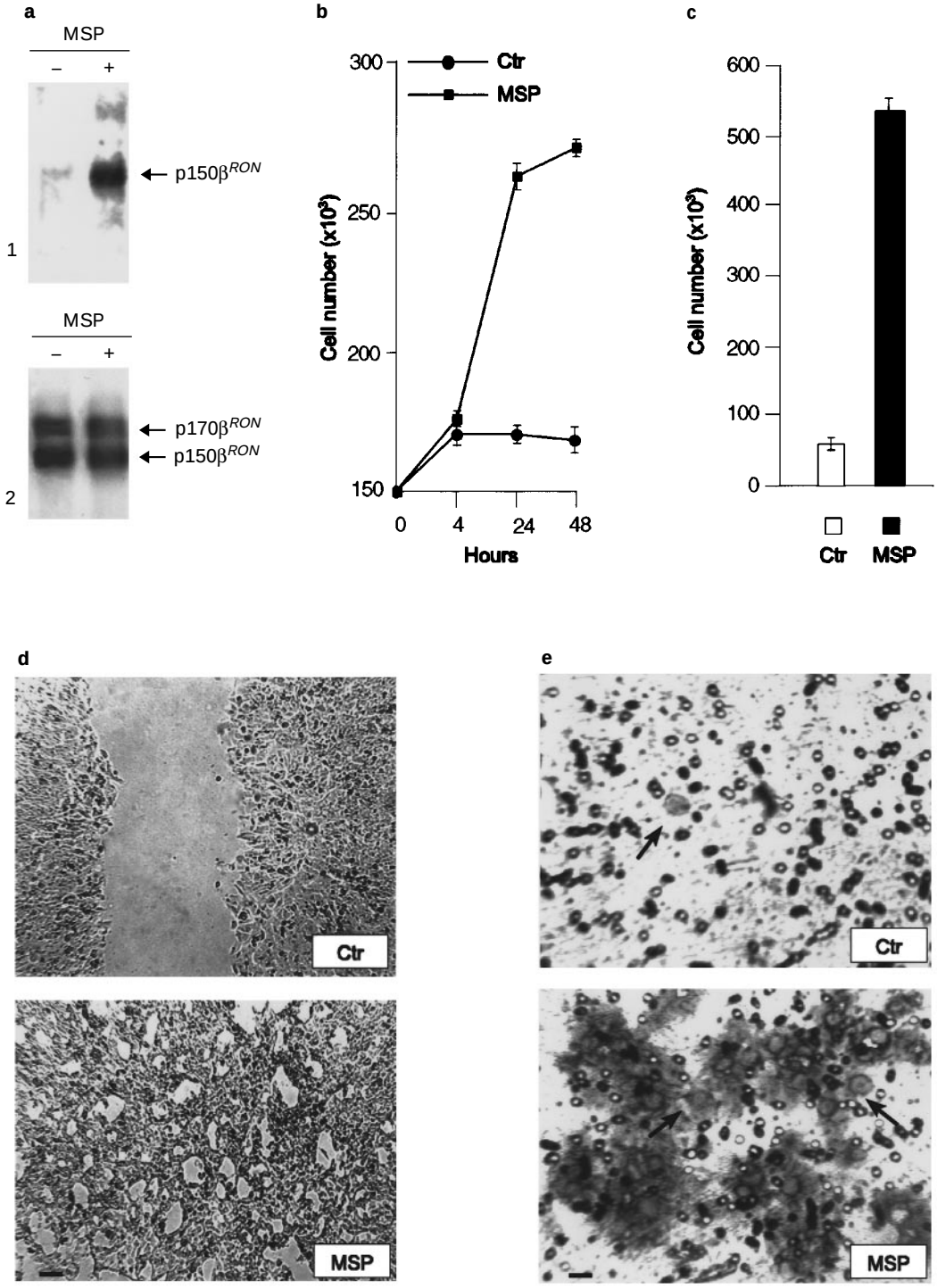

Figure 5 Biochemical and biological responses of ZR75.1 breast carcinoma cells to MSP/SF2. (**a**) MSP/SF2 induces phosphorylation of the *Ron* receptor. Proteins were extracted from untreated (−) and MSP/SF2-treated (+) cells, immunoprecipitated with the anti-*Ron* antibody and probed with anti-phosphotyrosine antibody (1) and with the same anti-*Ron* antibody (2). Upon addition of 300 ng/ml of MSP/SF2 the *Ron* receptor was strongly phosphorylated on tyrosine residues. A constitutive low level of *Ron* phosphorylation was present in cells stimulated with control medium. (**b**) Activation of the *Ron* receptor induces cell proliferation. Cells were grown in DMEM 3% FCS and incubated in the presence (MSP) or in the absence (Ctr) of MSP/SF2 at the above concentration. Cells were counted at 4, 24 and 48 h. Data are the average of three independent experiments. Standard deviations are shown. (**c**) Activation of the *Ron* receptor induces cell migration. Cells were plated on the upper compartment of a Transwell chamber with (MSP) or without (Ctr) the addition of MSP/SF2 at the above concentration in the lower compartment. Cells migrating through the filter were counted after 24 h. Bars indicate the Standard Deviation of four independent experiments. (**d**) Activation of the *Ron* receptor induces 'wound healing'. A confluent monolayer of cells was wound and incubated in the presence (MSP) or in the absence (Ctr) of MSP/SF2 at the above concentration. The pictures show healings observed after 72 h. Space bar corresponds to 200 μm. (**e**) Activation of the *Ron* receptor induces an invasive phenotype *in vitro*. Cells with invasive capacity were identified by their ability to pass through an artificial basement membrane. The experiment was done in the presence (MSP) or in the absence (Ctr) of the above concentration of MSP/SF2. The photographs show cells (arrows) which crossed the filter. Space bar corresponds to 20 μm

tumor suppressor gene *TP53*, have been identified as indicators of aggressive behavior and as prognostic markers (Kovach *et al*., 1996; Hartmann *et al*., 1997). Other abnormalities occur in dominant oncogenes, such as those encoding tyrosine kinase receptors. The expression of various members of the *EGFR* family, namely *EGFR*, *ERBB-2* and *ERBB-3* has been found overexpressed in a significant percentage (20–40%) of breast carcinomas (Sainsbury *et al*., 1985; Fox *et al*., 1994; Slamon *et al*., 1987) and in the case of *EGFR* and *ERBB-2* this expression correlates with poor prognosis (Toi *et al*., 1994; Slamon *et al*., 1987). However, both *TP53* mutation and overexpression of the *EGFR* family members have been mostly related to loss of growth control rather than cell invasive behavior, which still needs further study.

RON belongs to a tyrosine kinase gene family whose prototype is *MET*. A number of reports suggest that members of this family are involved in the progression of cancer: (I) germline and somatic missense mutations in the tyrosine kinase domain of *MET* are involved in papillary renal carcinogenesis (Schmidt *et al*., 1997); (II) *Met* is overexpressed in some carcinoma types at advanced stages (Di Renzo *et al*., 1995); (III) activation of the *Met* receptor via an experimental autocrine circuit (Rong *et al*., 1994) or by rearrangement (Giordano *et al*., 1997) confers metastatic properties; (IV) all the known members of the family stimulate invasion *in vitro* (Giordano *et al*., 1993; Medico *et al*., 1996); (V) although no transforming counterpart of the *RON* gene has been identified so far, constitutive activation of the *Ron* kinase elicits a motogenic-invasive phenotype (Collesi *et al*., 1996; Santoro *et al*., 1996).

In the present study we show, for the first time, that the *RON* gene is associated with a human cancer. The *Ron* protein, barely detectable in normal breast epithelium and in benign lesions (from calcifications to non-invasive breast tumors), is overexpressed in a significant percentage of infiltrating carcinomas.

This aberrant expression may be due to amplification or structural alteration of the gene. To assess whether an amplicon was present in the DNA, we analysed the genomic region where *RON* and *MSP/SF2* are located: the short arm of chromosome 3 (locus 3p21, Ronsin *et al*., 1993; Han *et al*., 1991). We did not find any amplification in this region. This data suggests that overexpression is controlled at transcriptional level. Interestingly, the *RON* promoter contains different putative binding sites for members of the Ets transcription factor family, which have been associated with the induction of the invasive-metastatic phenotype (Wernert *et al*., 1994) and are known to be powerful inducers of *MET* expression (Gambarotta *et al*., 1996). This suggests that, in cancer cells, genes of the *MET* family might be deregulated by a similar transcriptional mechanism.

From a clinical point of view, tumors overexpressing *RON* were detected in a significant number of postmenopausal patients. This information is puzzling as *Ron* expression does not relate to the steroid hormone receptor status of tumors and no canonical Steroid Receptor Elements have been found in the *RON* promoter (Del Gatto *et al*., 1995). Although the mechanism is still unknown, this clinical correlation suggests that *RON* gene overexpression contributes to the development of tumors in a subset of patients.

A number of studies have demonstrated that the protein tyrosine kinase activity might be significantly higher in most malignant human breast cancers than that in normal tissue or in benign tumors (Hennipman *et al*., 1989). Here we show that the overexpression of the *Ron* receptor in human breast cancers is accompanied by its phosphorylation. *Ron* autophosphorylation could result from clustering and consequent intermolecular interactions of $p150\beta^{RON}$ monomers, concentrated at high densities at the plasma membrane, or from binding of its own ligand (MSP/SF2) to the external domain of the receptor. Currently no information is available on the involvement in carcinogenesis of this factor. MSP/SF2 might act in a paracrine fashion or via an autocrine mechanism, as demonstrated for the *Met/HGF* complex in human and murine mammary cancer cells (Tuck *et al*., 1996; Rahimi *et al*., 1996).

Whatever the mechanism is, our results provide evidence that the overexpressed *Ron* receptor is functionally active in primary breast cancer. The possible biological role of the *RON* gene in the progression toward malignancy of breast cancer cells was analysed *in vitro*. In the model cell line ZR75.1, the *Ron* receptor is functionally active as it is phosphorylated upon the addition of its specific ligand and transduces signals which elicit cell growth, motility, migration and invasiveness. Altogether these results suggest that this receptor might confer to breast cancer cells selective properties to move and invade the basement membrane after stimulation by MSP/SF2.

We found that *RON* is expressed at high levels in the majority of the human breast cancer cell lines analysed. Interestingly, in these cell lines the expression of *Ron* inversely correlates with the expression of *Met* (Stoker *et al*., 1987; Byers *et al*., 1994). The data suggesting a role for *Met*/HGF complex in breast cancer progression are conflicting (Yamashita *et al*., 1994; Nagy *et al*., 1995; Lin *et al*., 1996; Tuck *et al*., 1996). Here, we show that another member of the *MET* family, with similar biological properties, is overexpressed in a large percentage of breast carcinomas. This observation, together with the demonstration that *Ron* receptor mediates invasiveness in breast tumor cells in response to its ligand, led us to speculate that this receptor may have a critical role in the progression of mammary carcinomas.

Materials and methods

Reagents and cell lines

Full length pro-MSP/SF2 cDNA was cloned in the Baculovirus transfer vector pVL1392 (Invitrogen) and transfected into *Spodoptera frugiperda* cells (Sf9 cells) along with Bsu36 I-digested BacPAK6 viral DNA using Lipofectin method (GIBCO BRL). Conditioned supernatant of Sf9 cells was collected 10 days later and tested for MSP/SF2 activity performing a *Ron* phosphorylation assay on T47D cells. Single recombinant MSP-Baculovirus plaques were isolated by standard plaque assay. Fifteen single plaques were tested for MSP/SF2 activity as described above and one of them was used to produce the pro-MSP/SF2 required for all the experiments. Recombinant pro-MSP/SF2 was cleaved to the mature form MSP/SF2 by incubation at 37°C for 12 h, in the presence of 10% Fetal Calf Serum (FCS). Conditioned

medium derived from Sf9 cells infected with an empty pVL1392-BacPAK6 virus added of 10% FCS was used as control in all the experiments described in this work.

Breast cancer cell lines ZR75.1, BT.474, MDA-MB.231, SK-BR.3, MCF.7 were purchased from the ATCC. GTL-16 gastric cancer cell line has been previously characterized (Giordano *et al*., 1989). Cells were grown in DMEM supplemented with 10% FCS in a humidified atmosphere with 5% CO_2.

Tissue samples

Breast tissues were from patients treated at the Department of Gynecologic Oncology of the University of Torino Medical School and Department of Endocrinology of the University of Catania Medical School. Carcinomas (74 cases) were classified as ductal (57) or non-ductal (17). In 67 cases pTNM and Grading were available as follows: T1 = 29 cases, T2 – 4 = 38 cases; G1 – G2 = 39 cases, G3 = 28 cases; ER + = 46, ER – = 28; PR + = 42, PR – = 32. Normal breast tissue was obtained from reduction mammoplasty.

Statistics

The correlation of various disease parameters was evaluated using contingency tables analysed by the χ^2 test. A P value of <0.05 was considered to be significant. When the sample size was relatively small the Fisher exact test was used to calculate the differences between groups in contingency tables. Pearson or Spearman correlation coefficient were calculated.

Immunoprecipitation and Western blot analysis

Tissue samples, removed at surgery, were treated as previously described (Di Renzo *et al*., 1994). The powdered whole tissue was homogenized in TEN buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1% Nonidet P40, 0.5% NaDOC), containing protease inhibitors. Equal amounts of proteins were immunoprecipitated with a rabbit polyclonal antibody raised against the C-terminal tail of human *Ron* (Gaudino *et al*., 1994). SDS–PAGE, Western blot with streptavidin-HRP and enhanced Chemiluminescence kit (ECL, Amersham) were performed. Under reducing conditions, the 150 KDa β and the 35 KDa α chain which constitute the 185 KDa *Ron* receptor are separated. The *Ron* antibody recognizes the β chain and the 170 KDa precursor. Using a RIPA buffer only the β chain was detectable. The expression of *Ron* protein was quantified by laser densitometric scanning of X-ray films and normalized by comparison with the control sample included in each gel (GTL16 extracts). The relative number of epithelial cells in each sample was analysed performing a Western blot on total proteins. Blots were then decorated with a K19 cytokeratin monoclonal antibody (Amersham).

Tyrosine phosphorylation assay

In vivo tyrosine phosphorylation of breast tissues and cells was evaluated by *Ron* immunoprecipitation and Western blot analysis using phosphotyrosine monoclonal antibodies (UBI). Tumor tissues were snap frozen in liquid N_2 immediately after surgery and stored at $-70^{\circ}C$ for a maximum of 3 weeks, in order to maintain the phosphorylation state of the proteins. Subconfluent ZR75.1 cells were treated or untreated with recombinant MSP/SF2, for 10 min at 37°C. Tissues and cells were lysed in TEN buffer containing 100 μM Na_3VO_4 and protease inhibitors. Immunoprecipitation was performed as described above.

Immunohistochemistry

Surgical specimens were fixed and paraffin embedded by standard procedures. Four μm sections from wax tissue blocks were deparaffinized and immersed in a 0.1% hydrogen peroxide-methanol solution to block the endogenous peroxidase activity. Sections were covered overnight at room temperature with Ron polyclonal antibody (Santa Cruz), followed by incubation with avidin-biotin-peroxidase complex (ABC, Vector Labs) for 30 min at room temperature. After incubation with a solution of 0.01% diaminobenzidine and hydrogen peroxide for 5 min, the sections were lightly counterstained with haematoxylin.

Biological assays

Cell proliferation was tested as follows: 1.5×10^5 cells/20 mm culture dish were plated in DMEM 10% FCS for 24 h, added of MSP/SF2 (300 ng/ml) in DMEM 3% FCS and counted after 4, 24 and 48 h.

For the wound healing assay 1.5×10^6 cells were seeded into 35 mm plate wells and grown for 48 h. A wound was made with a plastic pipette tip in a confluent monolayer of cells. Cells were then incubated for 72 h in the presence of DMEM 3% FCS, with or without MSP/SF2 at the above concentrations, fixed with glutaraldehyde and stained with crystal violet. Images of cell samples were taken by a computer assisted telecamera.

The assays for cell motility and matrix invasion were performed in Transwell® chambers (Costar Corporation). Briefly, 10^5 cells seeded on the upper side of a porous polycarbonate membrane (8.0 – μm – pore size) coated (invasion assay) or not coated (cell migration assay) with the artificial basement membrane Matrigel® (12.5 μg per filter; Collaborative Biomedical Products; Becton Dickinson Labware). MSP/SF2 at the above concentrations was added to the lower compartment. Only cells able to migrate or with an invasive phenotype, move through the membrane and grow on the lower side of the filter. After 24 h of incubation (for migration assays) or 72 h (for invasion assays), cells attached to the upper side of the filters were mechanically removed, whilst cells that migrated or invaded the Matrigel® were fixed and stained as previously described (Medico *et al*., 1996). Cells were counted (migration assay) or cells were photographed by a computer assisted telecamera (invasion assay). Both assays were performed in the presence of low serum concentration (3% FCS).

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