Expression of the c-Met/HGF receptor in human melanocytic neoplasms: demonstration of the relationship to malignant melanoma tumour progression

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Summary The c-MET proto-oncogene encodes the receptor for the Hepatocyte Growth Factor/Scatter Factor, which is known to mediate mitogenic, motogenic and invasive responses of several cell types. We have analysed by immunohistochemistry and biochemically the expression of c-MET in benign and malignant melanocytic lesions. The Met/HGF receptor which in the melanocytic lineage displays the structural features of the authentic receptor was undetectable in tissue melanocytes and in nevocytic nevi. Only four out of 23 primary melanomas scored positive. Expression increased to a significant level in 17 out of the 44 metastatic lesions examined. The c-MET expression was homogeneous in multiple metastases from the same patients. Comparative analyses showed both lack of correlation with the expression of the tumour progression associated ICAM-1 adhesion molecule and, in 23% of cases, co-expression with the c-KIT encoded receptor. These findings show that the c-MET gene is expressed at late stages of melanoma progression and suggest that the presence of Met/HGF receptor may contribute to the acquisition of an invasive phenotype.

The c-MET proto-oncogene encodes a transmembrane tyrosine kinase identified as the receptor for a polypeptide known as Hepatocyte Growth Factor (HGF) (Bottaro et al., 1991; Naldini et al., 1991a; Naldini et al., 1991b). HGF (Miyazawa et al., 1989; Nakamura et al., 1989; Zarnegar et al., 1989) is indistinguishable from Scatter Factor (SF), originally identified as a powerful polypeptide factor stimulating epithelial cell motility (Stoker et al., 1987; Weidner et al., 1990; Gherardi et al., 1989). HGF/SF has been shown to exert a pleiotropic activity on several cell types mainly of epithelial origin. It is a powerful mitogen for hepatocytes both in vitro and in vivo (for a review see Gherardi and Stoker, 1991) and stimulates the growth in vitro of several other epithelial cells, including kidney tubular cells, keratinocytes and endothelial cells (Kan et al., 1991; Busolino et al., 1992; Rosen et al., 1990; Rubin et al., 1991). Interestingly, HGF/SF is a powerful inducer of epithelial cell dissociation, able to increase their motility and invasiveness (Weidner et al., 1990). A role of HGF/SF and its receptor in promoting growth of tumour cells and/or in influencing their metastatic behaviour has been proposed (for a review see Comoglio, 1993). It has been recently shown that the MET encoded HGF/SF receptor is overexpressed in carcinomas of the GI tract (Di Renzo et al., 1991) and in thyroid carcinomas belonging to clinically and histologically advanced subtypes (Di Renzo et al., 1992). All this pointed to the involvement of HGF/SF and its receptor in the progression of tumour cells to a more malignant phenotype.

HGF/SF has been found to be mitogenic for melanocytes in primary cultures, mainly in the presence of synergistic factors (Matsumoto et al., 1991; Rubin et al., 1991; Halaban et al., 1992) and its receptor has been detected in human melanocytes and melanomas grown in vitro (Kan et al., 1991; Halaban et al., 1992). In this paper, we studied the expression of the Met/HGF receptor in the natural history of human melanocytic lesions, by examining benign nevi, primary melanomas and metastases. We show that the expression of immunologically detectable Met/HGF receptor increases, with significant incidence (39%), in metastatic lesions, suggesting a correlation with the progression of melanoma.

Material and methods

Tissue specimens and cell lines

Surgical biopsies of benign, malignant primary and metastatic melanocytic lesions were obtained from the Surgical Pathology section of the Regina Elena Cancer Institute. Tissue samples were snap frozen in liquid nitrogen. From each specimen 4 μm cryostat sections were obtained which were fixed in absolute acetone for 10 min. Fixed sections were either immediately used in immunohistochemical assays or kept frozen at –70°C with no loss of serological activity. Fixed sections stained with 1% toluidine blue were used to evaluate the histological features of the lesions. Tumour thickness was evaluated according to Breslow (1970). Primary cultures of cutaneous melanocytes were obtained from Clonetics (San Diego, USA). Cytospins were prepared using a Shandon cytocentrifuge (Runcorn, Cheshire, UK).

Monoclonal antibodies

The murine monoclonal antibody DQ13 against the α chain of Met/HGF receptor (Prat et al., 1991a) was raised against a peptide corresponding to nineteen c-terminal amino acids (from Ser137 to Ser266) of the c-Met sequence, EMBL DataBank reference n.X54559. The murine mAb DO24 to an epitope of the extracellular domain of the c-met gene product was produced using the human gastric carcinoma cell line GTL-16 as immunogen (Prat et al., 1991b). MoAb 84h10 to the intercellular adhesion molecule ICAM-1 was obtained from Immunotech, Marseille, France. MoAb to the extracellular domain of the c-kit receptor was purchased from Boehringer Mannheim.

Serological assays

The immunohistochemical analysis employed MoAb DO24 as purified reagents (Russo et al., 1983) at concentrations ranging from 10 to 50 μg ml⁻¹. The indirect immunoperoxidase stain was performed with commercially available reagents (Vectorstain Elite, Burlingame, CA, USA). Slides were incubated overnight with MoAb at 4°C in a moist chamber. The enzymatic activity was developed using 3- amino-9-ethylcarbazole (AEC) as chromogenic substrate for 8 min. Slides were then rinsed with phosphate buffered saline and counterstained with Mayer's haematoxylin. Sections on

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which the incubation of the primary antibody was omitted were used as control.

**Western Blotting**

Surgical biopsies immediately frozen in liquid nitrogen were pulverised using a Mikro-Dismembrator TM (B-Braun) in the presence of liquid nitrogen. Powdered tissues were solubilised in boiling Laemmli buffer (Laemmli, 1970), containing the reducing agent β-mercaptoethanol. Four hundred μg of proteins were loaded on each lane. Western blot analysis was carried out as described by Towbin et al. (1979).

Bound antibodies were revealed with rabbit anti-mouse antibodies labelled with HR Peroxidase according to the enhanced chemiluminescence method (ECL™, Amersham).

**Results**

Table I shows the list of melanocytic samples examined by immunohistochemistry. Cultured melanocytes, nevus cells of intra-dermal and junctional nevi were not labelled by the monoclonal antibody directed against the extracellular domain of the MET/HGF receptor (Figure 1a). In the same biopsies MoAb DO24 stained with variable intensity the keratinocytes of the basal layer as well as melanophages. Similarly the receptor was not histochemically detectable in the majority of primary tumours (Table I). However four lesions were scored positive, showing either a homogenous-weak staining or an irregular distribution of the antigen. The four positive tumours displayed a high degree of dermal invasiveness (Figure 1b). For comparison the pattern of expression in the same lesions of the ICAM-1 molecule which is known to be associated with increasing tumour invasiveness (Natali et al., 1990) is reported (Table II). Positive staining with the ICAM-1 monoclonal antibody was observed with the expected distribution without a significant correlation with the expression of the MET/HGF receptor. In contrast to primary tumours 17 out of 44 (39%) metastatic foci collected from different body sites, showed a positive staining with an apparent lower incidence in parenchymal metastases. The intensity of the stain was generally weak and only in a minority of the instances was homogenously distributed in the metastatic cell population (Figure 1c). Evaluation in four patients of individual concomitant metastases (Table III) showed that the expression of the gene product is rather consistent among autologous lesions. Because the expression of the c-kit receptor for Stem Cell Factor (SCF) has been shown to be downregulated in human melanocytes following malignant transformation (Natali et al., 1992) a comparative analysis of the c-met and c-kit products in 16 unselected metastatic lesions was performed. The results reported on Table IV demonstrated that the two receptor are uncoordinately expressed with only 20% of the samples displaying detectable levels of both molecules. Furthermore no relationship was found between expression of c-met and degree of pigmentation. In order to investigate the molecular structure of the c-met receptor expressed by melanoma cells, selected biopsies expressing different levels of c-met were also analysed by Western blotting employing MoAb DQ13 against the COOH-terminal tail of the human protein. Figure 2 shows that the MET/HGF receptor of melanocytic lesions has structural features of the authentic receptor. The levels of expression of the MET/HGF receptor detected in Western blot analysis corresponded to those detected by immunohistochemistry.

| Table I Immunohistochemical detection of Met/HGF receptor in benign and malignant lesions of the melanocyte lineage |
|---------------------------------------------------------------------------------------------------------|
| Melanocytes (primary cultures)                                                                         | neg. |
| Intra-dermal-junctional nevi                                                                            | 0/15^a |
| Blue nevi (simple and cellular type)                                                                    | 0/5  |
| Primary melanomas:                                                                                    |      |
| melanoma in situ                                                                                       | 0/3  |
| melanoma from superficial spreading                                                                   | 3/12 |
| nodular melanoma                                                                                       | 1/6  |
| acral lentiginous melanoma                                                                             | 0/2  |
| total                                                                                                 | 4/23 |
| Metastatic melanomas:                                                                                 |      |
| lymphomonalytic                                                                                       | 13/33|
| cutaneous                                                                                             | 3/7  |
| parenchymal                                                                                           | 1/4  |
| total                                                                                                 | 17/44|

^a No. positive/no. tested.
Table II Immunochemistry detection of Met/HGF receptor and ICAM-1 adhesion molecule in primary melanomas of increasing dermal invasiveness

| Case | Histotype (thickness: mm) | MET receptor | ICAM-1 |
|------|--------------------------|--------------|--------|
| BA   | MSS (1.0)                | -            | var    |
| TI   | MSS (1.5)                | -            | -      |
| PO   | MSS (1.9)                | -            | -      |
| OR   | MSS (2.0)                | -            | -      |
| CA   | MSS (2.0)                | -            | -      |
| DN   | MSS (2.0)                | -            | -      |
| CF   | NM (2.7)                 | +            |        |
| AL   | NM (3.2)                 | -            | -      |
| GI   | NM (3.2)                 | -            | +      |
| BE   | NM (4.0)                 | -            | var    |
| OP   | NM (4.5)                 | var          | var    |
| BO   | MSS (4.5)                |              | var    |
| PR   | MSS (4.5)                | +            | is     |
| TA   | ALM (4.5)                | -            | +      |
| BY   | NM (5.0)                 | -            | +      |
| FI   | MSS (6.0)                |              | -      |
| PL   | ALM (>6.0)               | -            | +      |

*MSS: melanoma from superficial spreading, NM: nodular melanoma, ALM: acral lentiginous melanoma. ±: no stain, -: homogeneous weak stain, +: homogeneous stain, var: stain of variable intensity from negative to positive, is: isolated areas stained accounting for less than 20% of the lesion.

Table III Immunochemistry detection of Met/HGF receptor in concomitant autologous lymphonodal melanoma metastases

| Patient | Metastasis no. 1 | Metastasis no. 2 | Metastasis no. 3 | Metastasis no. 4 |
|---------|------------------|------------------|------------------|------------------|
| PI      | neg.             | neg.             | neg.             | neg.             |
| PA      | var              | var              | var              | var              |
| BI      | neg.             | neg.             | neg.             | neg.             |
| IA      | var              | var              | var              | var              |

neg: no stain, var: stain of variable intensity.

Table IV Immunochemistry detection of Met/HGF receptor and Kit/SFC receptor in metastatic melanomas

| Patient | Site          | Pigmentation | MET/HGF | KIT/SFC |
|---------|---------------|--------------|---------|---------|
| 1       | CEC Lymphnode | ±            | var (50%)| var (20%)|
| 2       | COR Lymphnode | -            | var (50%)| -       |
| 3       | COP Lymphnode | -            | -       | -       |
| 4       | PAL Subcutis  | +            | -       | +       |
| 5       | BON Lymphnode | +            | -       | -       |
| 6       | PET Lymphnode | -            | -       | var (80%)|
| 7       | PIE Subcutis  | -            | var     | -       |
| 8       | LOM Subcutis  | ±            | var (30%)| var (20%)|
| 9       | CAS Lymphnode | -            | var     | -       |
| 10      | CAR Lymphnode | -            | -       | -       |
| 11      | MAN Subcutis  | ±            | -       | +       |
| 12      | MAY Lymphnode | +            | -       | var     |
| 13      | STE Liver     | -            | -       | -       |
| 14      | ALL Lymphnode | + +          | var     | +       |
| 15      | VIN Lymphnode | -            | ±       | var     |

*: absence, ±: scattered, +: weak, + +: intense pigmentation. ±: no stain, var: staining of variable intensity, +: homogenous stain, ++ (%): intense stain (percentage of stained areas).
melanocyte lineage, showing that a consistent fraction of metastatic melanomas displays an increased level of expression of the MET/HGF receptor.

By immunohistochemistry employing MoAbs against the extracellular domain of the protein, the receptor was undetectable in melanocytes in benign nevic lesions and in the majority of the primary melanomas of increasing dermal invasiveness. Its role in the control of proliferation of melanocytes and primary melanomas cannot be excluded, since very low levels of the receptor (120 molecules/cell) (Matsumoto et al., 1991) below the sensitivity of the immunohistochemical assay may be expressed in some lesions. The positive control stain of keratinocytes and of melanophages in the same tissue samples demonstrated that the receptor can be detected immunohistochemically when expressed at a higher level. Despite the low levels of receptor, MET/HGF has been shown to promote proliferation and motility in normal melanocytes as well as to maintain high levels of tyrosinase activity i.e. pigmentation (Halaban et al., 1992). We have shown that increased expression of the MET/HGF occurs in a significant fraction of metastatic melanoma, but without correlation with the degree of pigmentation, thus indicating that this control function of c-met may be impaired in metastatic cells.

The rather homogenous expression of the receptor in multiple autologous metastases suggests that during tumour progression a selection of a melanoma cell subpopulation with a constitutive capacity to synthesise the receptor may occur, thus conferring a potential drive to metastasise. While per se motogenic, MET/HGF is mitogenic in the presence of synergistic factors such as bFGF and SCF (Halaban et al., 1992). The demonstration in our study of the uncoordinated expression of c-met and c-kit as well as the lack of correlation of c-met expression and anatomical sites of the metastases (i.e. stromal relationship), suggest that the MET/HGF receptor may deliver mainly a motogenic stimulus to melanoma cells. It must be recalled in this context that the expression of a functional MET/HGF receptor has been shown to be sufficient to confer on cells an invasive phenotype. In presence of HGF/SF, NIH-3T3 fibroblasts, transfected with the cMET proto-oncogene, are prompted to invade collagen matrices and to migrate in Boyden chambers (Giordano et al., 1993). HGF/SF is widely distributed in tissues, bound to the extracellular matrix mainly in the precursor form (pro-HGF) activated by the widespread urokinase-e-type plasminogen activator (Naldini et al., 1992). A paracrine interaction may therefore be envisaged. The limiting step for melanoma progression towards the metastatic phenotype may thus be, in some instances, the expression of the MET/HGF receptor.

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