The Heregulin/Human Epidermal Growth Factor Receptor as a New Growth Factor System in Melanoma with Multiple Ways of Deregulation

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In a screening for new growth factors released by melanoma cells, we found that the p185-phosphorylating capacity of a medium conditioned by a melanoma cell line was due to the secretion of heregulin, a ligand for the human epidermal growth factor receptor (HER) family of receptor tyrosine kinases. Expression of heregulin, including a new isoform, and secretion of functionally active protein was found in several cell lines. Receptor activation by heregulin, either autocrine or paracrine, resulted in a potent growth stimulation of both melanocytes and melanoma cells. Heregulin receptor HER3 and coreceptor HER2 were the main receptors expressed by these cells. Nevertheless, none of the cell lines in our panel overexpressed HER2 or HER3. In contrast, loss of HER3 was found in two cell lines, whereas one cell line showed loss of functional HER2, both types of deregulations resulting in unresponsiveness to heregulin. This implies the heregulin/HER system as a possible important physiologic growth regulatory system in melanocytes in which multiple deregulations may occur during progression toward melanoma, all resulting in, or indicating, growth factor independence. ***Key words: heregulin-neuregulin-1/autocrine-paracrine communication/receptor tyrosine kinases.*** ***J Invest Dermatol 121:802–812, 2003***

Growth of melanocytes and their malignant counterparts is regulated by a variety of cytokines and other polypeptides (Lázár-Molnár *et al*, 2000; Payne and Cornelius, 2002). Under physiologic conditions, melanocytes depend for their survival on paracrine stimulatory factors provided by the surrounding keratinocytes (Meier *et al*, 1998). Transformed melanocytes have a decreased dependence on paracrine stimulation, which facilitates their survival outside their natural environment, the epidermis. Changes in several growth factor systems contribute to this decreased dependence. Whereas overexpression of receptor tyrosine kinases (RTK) may lead to increased growth factor sensitivity and constitutive signaling, loss of expression may result in insensitivity to inhibitory factors or indicate growth factor independence (Easty and Bennett, 2000). Also, the profile of growth factors secreted by melanoma cells is frequently altered, compared to melanocytes. Whereas *de novo* expression of some growth factors by melanoma cells may stimulate proliferation of these cells in an autocrine loop, these factors may act on the surrounding cells as well, stimulating or inhibiting these cells in a paracrine way (Halaban, 2000; Lázár-Molnár *et al*, 2000; Ruiter *et al*, 2002).

The human epidermal growth factor receptor (HER) family of RTK consists of four members, epidermal growth factor receptor (EGFR)/erbB1/HER1, neu/erbB2/HER2, erbB3/HER3, and erbB4/HER4 (Olayioye *et al*, 2000; Yarden and Sliwkowski, 2001). Although constitutive activation of these receptors, owing to overexpression, frequently occurs in various types of cancers (Révillion *et al*, 1998), this does not seem to be common in melanoma (Natali *et al*, 1994; Korabiowska *et al*, 1996; Persons *et al*, 2000; Fink-Puches *et al*, 2001). Constitutive RTK signaling may also be the result of truncation, mutation, association with other cell-surface proteins, transactivation via other receptors, or the presence of autocrine loops (Blume-Jensen and Hunter, 2001; Gullick, 2001). The latter may result from the aberrant expression of HER ligands.

Neuregulin-1 is the term for a family of proteins derived by alternative splicing from a single gene, functioning as ligand for HER3 and HER4 (Holmes *et al*, 1992; Yarden and Sliwkowski, 2001). At present, at least 24 splice variants have been identified in different species, of which 10 were found in humans. Alternative splicing at the N-terminus results in three types of proteins: heregulins (HRG, type I) (Holmes *et al*, 1992), glial growth factors (type II) (Marchionni *et al*, 1993), and sensory- and motor-neuron-derived factors (type III) (Ho *et al*, 1995). Further alternative splicing of HRG at the EGF-like domain (α or β), the C-terminal part of the EGF-like domain (1, 2, or 3) (**Fig 1**), and/or at the intracellular tail (a, b, or c) gives rise to closely related proteins, differing in size and cellular localization and having distinct receptor activation potentials and functions (Wen *et al*, 1994; Pinkas-Kramarski *et al*, 1996; Meyer *et al*, 1997). Transmembrane

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Abbreviations: bFGF, basic fibroblast growth factor; CM, conditioned medium; EGF, epidermal growth factor; HER, human EGF receptor; HRG, heregulin; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; rHRG-β1, 7-kDa recombinant EGF-like domain of heregulin isoform b1; RTK, receptor tyrosine kinase.

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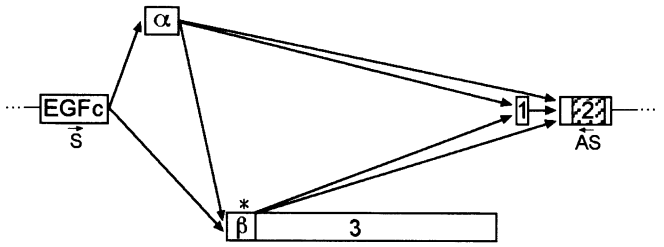


Possible exon combinations	Isoform	Expected PCR product length
(EGFc) - (α) - (2)	α2	202 bp
(EGFc) - (α) - (1) - (2)	α1	226 bp
(EGFc) - (β) - (2)	β2	193 bp
(EGFc) - (β) - (1) - (2)	β1	217 bp
(EGFc) - (α) - (β) - (2)	?	261 bp
(EGFc) - (α) - (β) - (1) - (2)	α4	285 bp
(EGFc) - (α) - (β) - (3)	α3	NA
(EGFc) - (β) - (3)	β3	NA

Figure 1. Neuregulin-1 splicing variation in the EGF-like domain. The scheme depicts the genomic organization of the exons encoding the region surrounding the variable part of the EGF-like domain of the NRG-1 gene. The locations of the sequences that are complementary to the primers used for RT-PCR are indicated with *S* and *AS* for the sense and antisense primers, respectively. The table indicates all possible splicing variants within this domain with their expected amplification product lengths in base pairs (bp), when using the indicated primer pair. *NA,* not amplified; *EGFc,* sequence common in the EGF-like domain of all HRG; *hatched,* coding sequence for the HRG transmembrane domain; *asterisk,* location of the stop codon in case of the (α)–(β) combination; *?,* putative isoform not yet characterized.

HRG typically function as precursor molecules that are subject to the action of metalloproteinases. This results in the release of the extracellular domain that may subsequently bind to nearby receptors (autocrine/paracrine action) (Montero *et al*, 2000; Shirakabe *et al*, 2001). Necessary and sufficient for receptor binding is the EGF-like domain; the roles of the various other domains have not been fully elucidated yet. Some regulation may be exerted by the cytoplasmic tail (Liu *et al*, 1998a, b; Han and Fischbach, 1999) or by interaction of the N-terminal heparin-binding motif with other molecules such as cell surface heparan sulfate proteoglycans (Li and Loeb, 2001). A recently proposed model for ligand-mediated HER activation proposes receptor conformational changes as the driving force for receptor activation (Cho and Leahy, 2002; Garrett *et al*, 2002; Ogiso *et al*, 2002). For HRG, this would mean that binding of its EGF-like domain to HER3 or HER4 leads to an altered receptor conformation, thus promoting dimerization with another HER, preferentially HER2. Hetero- or homodimerization of the receptors leads to *trans*- and autophosphorylation, creating specific docking sites for signal transduction molecules (Dankort *et al*, 2001; Hellyer *et al*, 2001) and initiating further downstream signaling. When only HER2 and HER3 are present, this model of HRG-induced receptor activation implies that HER3, which lacks catalytic activity (Guy *et al*, 1994; Sierke *et al*, 1997), can only become phosphorylated *in trans* by heterodimerization with HER2 (Kim *et al*, 1998). Conversely, HER2, for which no direct ligand has been identified yet, only becomes activated after ligand binding to HER3.

Based on the initial observation that conditioned medium (CM) from a melanoma cell line induced a strong phosphorylation of HERs in MCF-7 mammary cancer cells, we decided to dissect the role of this putative ligand–receptor system in melanocytes and a panel of melanoma cell lines. Here, in 4 of a panel of 13 melanoma cell lines, we describe a number of deregulations in the HRG/HER system. Production and release of functionally active HRG in the medium were found in three cell lines and resulted in an autocrine loop in one case. Whereas exogenous HRG-stimulated growth of the majority of melanoma cell lines and melanocytes, three cell lines did not respond to HRG, owing to the absence of HER3 or owing to a functionally incompetent HER2.

MATERIALS AND METHODS

Cell lines The cell lines were obtained as follows: 530 and BLM melanoma cell lines from L. Van Kempen (University of Nijmegen, the Netherlands); A375 melanoma cell line from J. Hilkens (NKI, the Netherlands); Bowes melanoma from G. Opdenakker (Rega Institute, Belgium); DX3 and DX3azaLT5.1 melanoma cell lines from J. Ormerod (Imperial Cancer Research Fund, UK); FM3/D, FM3/p, FM45, and FM87 melanoma cell lines from J. Zeuthen (Danish Cancer Society, Denmark); HMB2, MeWo, and MJM melanoma cell lines from D. Rutherford (Rayne Institute, St Thomas Hospital, UK); MCF-7/6 mammary carcinoma cell line (further called MCF-7) from H. Rochefort (University of Montpellier, France); COLO-16 squamous skin carcinoma cell line and SK-BR-3 mammary carcinoma cell line from C. De Potter (Ghent University Hospital, Belgium); and MDA-MB-231 breast cancer cell line from American Type Culture Collection (Manassas, VA). Cell lines were routinely maintained in the following media (Gibco BRL, Belgium): RPMI 1640 (FM and COLO-16 cell lines), L15 (MDA-MB-231), 50% Dulbecco's modified Eagle's medium/50% Ham's F12 (MCF-7), or Dulbecco's modified Eagle's medium (all other cell lines). All media for routine culture contained 10% heat-inactivated fetal bovine serum (Greiner Bio-One, Belgium), 100 IU per mL penicillin, 100 μg per mL streptomycin, and 2.5 μg per mL amphotericin B. Epidermal melanocyte primary cultures were obtained from neonatal foreskins and established in M199 medium (Gibco BRL), supplemented with 2% fetal bovine serum, 10^{-9} M cholera toxin, 10 ng per mL basic fibroblast growth factor (bFGF), 10 μg per mL insulin, 1.4 μM hydrocortisone, and 10 μg per mL transferrin (all from Sigma, Belgium). Postprimary cultures were maintained in low-calcium (0.03 mM) M199 medium, supplemented with the same factors and 10% fetal bovine serum. The melanocytic origin of all melanoma cell lines was checked by immunocytochemistry using two antibodies against melanoma-specific proteins, HMB45 (Enzo Diagnostics, Farmingdale, NY) and NKI/C3 (Biogenex, San Ramon, CA). All melanoma cell lines were positive for at least one of these markers (data not shown). Because most of the experiments were carried out with Bowes melanoma cells, which were only positive for NKI/C3, additional electron microscopy was performed to confirm the presence of premelanosome-like structures in this nonpigmented cell line (data not shown).

Antibodies and reagents Primary antibodies used were: rabbit polyclonal anti-HER1, -2, -3, and -4 and anti-HRG precursor antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antitubulin (Sigma), mouse antiphospho-mitogen-activated protein kinase (MAPK; Westburg, the Netherlands), and antiphosphotyrosine antibody RC20 conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY). Goat anti-HRG-α and recombinant HRG-β1, consisting of the EGF-like domain of HRG (rHRG-β1, used at 10 ng/mL unless indicated otherwise), was purchased from R & D Systems (Abingdon, UK). Full-length recombinant HRG-β1 was obtained from Laboratory Vision (Fremont, CA), whereas heparin, PD168393 (used at 2 μM, unless indicated otherwise), and PD98059 were from Calbiochem (Darmstadt, Germany).

Preparation of CM Subconfluent monolayers were washed three times with phosphate-buffered saline (PBS), incubated for 24 h with serum-free medium, and washed again three times with PBS, followed by a 48-h incubation with serum-free medium. The latter medium was cleared from cells by 5 min centrifugation at $250 \times g$. The resulting supernatant was centrifuged for an additional 20 min at $2000 \times g$ to remove cell debris, filtered through a 0.2-μm filter, and stored at –20°C until use. To isolate the heparin-binding fraction from the CM, the latter was depleted from heparin-binding factors by triple precipitations with heparin beads (Bio-Rad, Hercules, CA). Elution of the heparin-binding fraction was done with 1 M NaCl, followed by desalting and dilution in fresh serum-free medium.

Western blotting and (immuno)precipitation All lysates were made of cells of approximately 90% confluence. For phosphorylation experiments, cells were washed three times with PBS, serum-starved overnight, washed again three times with PBS, and treated with serum-free medium for the indicated times. Before making all lysates, the cells were washed three times with PBS. Cells were lyzed with PBS containing 1% Triton X-100, 1% Nonidet P-40 (Sigma), and the following protease inhibitors: aprotinin (10 μg/mL), leupeptin (10 μg/mL) (ICN Biomedicals, Costa Mesa, CA), phenylmethylsulfonyl fluoride (1.72 mM), NaF (100 μM), $NaVO_3$ (500 μM), and $Na_4P_2O_7$ (500 μg/mL) (Sigma). After clearing the lysates, protein concentration was determined using Rc Dc protein assay (Bio-Rad), and samples were prepared such that equal amounts of protein were to be loaded. For immunoprecipitation, equal amounts of protein were first incubated with protein A–Sepharose (Amersham Pharmacia Biotech, UK) for 30 min. After discarding the beads, the supernatant was incubated with primary antibody for 3 h at 4°C, followed by incubation with the added protein A–Sepharose beads for 1 h. For heparin and streptavidin precipitations, cell lysates were incubated with heparin beads (Bio-Rad) or streptavidin beads (Sigma). Sample buffer (Laemmli) with 5% 2-mercaptoethanol and 0.012% bromophenol blue was added, followed by boiling for 5 min and separation of proteins by gel electrophoresis on a 8 or 12% polyacrylamide precast gel (Invitrogen, San Diego, CA) and transfer onto a nitrocellulose membrane (Amersham Pharmacia Biotech). Quenching and immunostaining of the blots were done in 5% nonfat dry milk in PBS containing 0.5% Tween 20, except for RC-20 and antiphospho-MAPK antibodies, where 4% bovine serum albumin in PBS containing 0.2% Tween 20 was used instead. The membranes were quenched for 1 h, incubated with primary antibody for 1 h, washed four times for 10 min, incubated with horseradish peroxidase-conjugated secondary antibody for 45 min, and washed six times for 10 min. Detection was done using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) as a substrate. To control for equal loading of total lysates, immunostaining with antitubulin antibody was performed routinely (not shown). Quantification of bands was done using Quantity-One software (Bio-Rad).

RT-PCR, cloning, and sequencing Total RNA was extracted from approximately 5×10^6 cells using the Qiagen RNeasy kit (Qiagen, Chatsworth, CA). One microgram of total RNA was reverse transcribed with oligo(dT) primers using the Qiagen RT kit (Qiagen) according to the manufacturer's instructions. HRG cDNA encoding all transmembrane isoforms was amplified using the sense primer 5′-CTGTGTGAATGGAG-GGGAGTGC-3′ (complementary to a sequence encoding a conserved part of the EGF-like domain) and the antisense primer 5′-GACCACAAG-GAGGGCGATGC (complementary to a sequence encoding part of the transmembrane domain) (**Fig 1**). As a control (not shown), β2-microglobulin cDNA was amplified using the sense primer 5′-CATCCAGCGTACTC-CAAAGA-3′ and the antisense primer 5′-GACAAGTCTGAATGCTC-CAC-3′ to generate a 165-bp product. PCR was performed on 250 ng template cDNA using the Qiagen *Taq* PCR kit (Qiagen) according to the manufacturer's instructions. Reactions were done in a Minicycler (Biozym, the Netherlands) with an initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 50 s (denaturation), 61°C for 50 s (annealing), and 72°C for 1 min (elongation), followed by a final extension at 72°C for 10 min. For cloning of the HRG amplification products, the HRG sense and antisense primers were extended at the 5′ end with GCCGGATCCG, creating a *Bam*HI restriction site, and with TCCGAATTC, creating a *Eco*RI restriction site, respectively. The resulting amplification products were either separated by agarose gel electrophoresis, followed by gel extraction using Qiagen gel extraction kit (Qiagen), or used directly for digestion with *Bam*HI and *Eco*RI restriction enzymes (Roche Diagnostics, Germany). Digested products were ligated into dephosphorylated *Bam*HI/*Eco*RI-digested pIRES2-EGFP vector (Clontech, Palo Alto, CA). After transformation of competent DH5α bacteria with the ligated product, the kanamycin-resistant clones were screened by PCR using primers complementary to sequences of the pIRES2-EGFP vector surrounding the insert. This resulted in PCR products of different lengths, corresponding to different HRG isoforms, which were subjected to sequencing (Applied Biosystems, Foster City, CA). The sequence of the α4-isoform was submitted to GenBank (Accession Number AY207002).

Scattering assay MCF-7 cells were seeded until small islands were formed. The cells were washed three times with PBS and were serum-starved overnight. The following day, the cells were washed again three times with PBS, after which the treatments (all in serum-free medium) were applied for 2 h. Pictures were taken with an Axiovert 200M microscope (Carl Zeiss Vision, Germany) on living cultures or after the cultures had been fixed with crystal violet (0.5% in 4% formaldehyde, 30% ethanol and 0.17% NaCl) for 15 min.

Cell proliferation assays A total of 12,500 melanocytes were seeded in the wells of a 96-well plate in 100 μL of Dulbecco's modified Eagle's medium/Ham's F12 medium containing 10% fetal bovine serum. After attachment, 100 μL of medium, supplemented with growth factors as indicated, was added. After 5 days, metabolic activity was measured with a colorimetric assay. Briefly, 100 μL of medium was taken off, followed by the addition of 20 μL of 5 mg per mL 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) in PBS. After a 2-h incubation and removal of all fluid, the colored formazan formed was dissolved in 100 μL of dimethyl sulfoxide and absorption was measured with an ELISA reader at 490 nm. Proliferation of melanoma and MCF-7 cells was measured with the sulforhodamine B colorimetric assay. A total of 5000 cells were seeded in 96-well plates, allowed to attach, and treated as indicated. After 5 days, 50 μL of 50% trichloroacetic acid was added to the culture medium, followed by an incubation of 1 h at 4°C. The wells were rinsed with water, dried, incubated with 100 μL of sulforhodamine B (0.4% in 1% acetic acid) for 30 min, rinsed with 1% glacial acetic acid, and dried again. Bound dye was dissolved in 200 μL of 10 mM Tris buffer, pH 10.5, and absorption was measured with an ELISA reader at 490 nm.

Annexin V staining Melanocytes were seeded on a collagen type I gel for 4 days. After taking photographs, adherent cells were detached with a swab and brought together with floating cells. Annexin V staining was performed with Annexin V–PE (Becton Dickinson Biosciences, Mountain View, CA), according to the manufacturer's instructions. The cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson) with an argon–ion laser tuned at 488 nm and a helium–neon diode laser at 635 nm. Forward light scattering, orthogonal scattering, and two fluorescence signals were stored in list-mode data files. Data acquisition and analysis were done using the CellQuest software (Becton Dickinson). Additional propidium iodide (PI) staining was performed to rule out cells that were necrotic (Annexin V^+ and PI^+).

Statistics Differences between means were considered significant when the p value was less than 0.01, using Student's *t* test.

RESULTS

Expression of HERs in a panel of melanoma cell lines Expression of HERs by melanocytes and melanoma cell lines was examined by Western blotting and immunostaining with anti-HER1, -2, -3, and -4 antibodies. Neither melanocytes nor any of the melanoma cell lines showed expression of full-length HER1 or HER4, compared with the respective positive controls A431 and T47D (data not shown). Nevertheless, this does not necessarily mean that these receptors are completely absent. All melanoma cell lines, as well as melanocytes, expressed HER2 (**Fig 2**). HER2 levels in melanocytes and in all

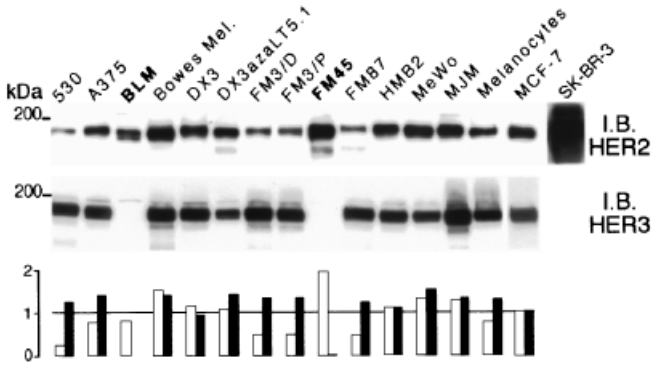


Figure 2. Analysis of HER2/HER3 expression in melanocytes and melanoma cell lines. Whole-cell lysates were analyzed by immunoblotting (*I.B.*) with anti-HER2 and anti-HER3 antibodies, as described under Materials and Methods. Quantification of the resulting bands was done relatively to the level of HERs in MCF-7 mammary carcinoma cells, set at 1. *Open and filled bars,* HER2 and HER3 expression, respectively.

melanoma cell lines were far below the level found in the HER2-overexpressing SK-BR-3 mammary carcinoma cell line (Press *et al*, 1993). Moreover, quantification showed that none of the melanoma cell lines had HER2 levels that were more than two times higher than that seen in MCF-7 mammary carcinoma cells, often used as a control for normal expression (Press *et al*, 1993; Aguilar *et al*, 1999). Comparable levels of HER3 were expressed by melanocytes and 11 of the melanoma cell lines. Two melanoma cell lines (BLM and FM45) did not show expression of HER3 protein (**Fig 2**). The absence of HER3 protein was due to a strongly reduced level of HER3 mRNA in these cell lines, compared with the other cell lines (data not shown).

Expression of HRG by melanoma cell lines Western blotting, followed by immunostaining of total lysates using an anti-HRG antibody directed against a cytoplasmic sequence conserved in all transmembrane HRG isoforms, revealed the presence of a ± 105-kDa band in Bowes melanoma, BLM, and MJM, the first two having the stronger expression (**Fig 3*A***, *top panel*). This band was also present in the positive controls MDA-MB-231 (although very faint) and COLO-16, previously described to secrete HRG (Holmes *et al*, 1992) or HRG-like activity (De Corte *et al*, 1994), respectively, and was not found in the MCF-7-negative control (Aguilar *et al*, 1999; Aguilar and Slamon, 2001). The size of this band indicates that it corresponds to the full-length HRG precursor (Burgess *et al*, 1995; Aguilar and Slamon, 2001). The 50-kDa band, also seen by others using this antibody (Aguilar and Slamon, 2001), probably represents an artifact, because it could also be found in the MCF-7 HRG-negative cells. Also two other bands (at ± 85 and 75 kDa), seen in some melanoma cell lines, are likely due to cross-reactivity of the antibody with other proteins. Because these bands were not consistently found in cell lines expressing HRG mRNA, and CM of these cells had no HRG-like activity (see below), they are unlikely to represent cleavage products of transmembrane HRG. The localization of the 105-kDa immunoreactive band at the plasma membrane was confirmed for Bowes melanoma cells by biotinylation (**Fig 3*B***, *lane 4*) and by precipitation using heparin beads (**Fig 3*B***, *lane 3*), which is consistent with the presence of a heparin-binding motif at the extracellular N-terminus of HRG.

RT-PCR analysis was carried out to verify the results obtained by western blotting and to detect which isoforms were expressed by the HRG-positive melanoma cell lines. Because alternative splicing of the HRG-encoding gene leads to multiple isoforms, with most variation in the EGF-like domain, primers were chosen so that different lengths of amplification products were obtained, depending on the isoform expressed. The sense and antisense primers chosen were complementary to the mRNA encoding a conserved part of the EGF-like domain and a sequence conserved in all transmembrane isoforms, respectively (**Fig 1**). RT-PCR using this primer set, with MDA-MB-231 and COLO-16 as positive controls and MCF-7 as a negative control, confirmed the results obtained by western blotting for Bowes melanoma, BLM, and MJM cells (**Fig 3*A***, *bottom panel*). In addition, lower levels of mRNA were found in some other melanoma cell lines, possibly resulting in HRG protein levels that were below the detection level in western blotting. Melanocytes had amounts of mRNA that were comparable to those found in MJM cells. Because we initially did not detect HRG protein in these cells (**Fig 3*A***), we loaded more protein and overexposed the film, which eventually resulted in the appearance of a weak band at ± 105 kDa (**Fig 3*C***). The pattern of PCR amplification products that was obtained from Bowes melanoma suggested the presence of multiple isoforms. Cloning and sequencing of these products revealed that α2, β1, and β2 isoforms were the most abundant transmembrane isoforms in this cell line (**Fig 3*D***). In addition, a new isoform, designated α_4, was identified. This isoform combines the sequences from

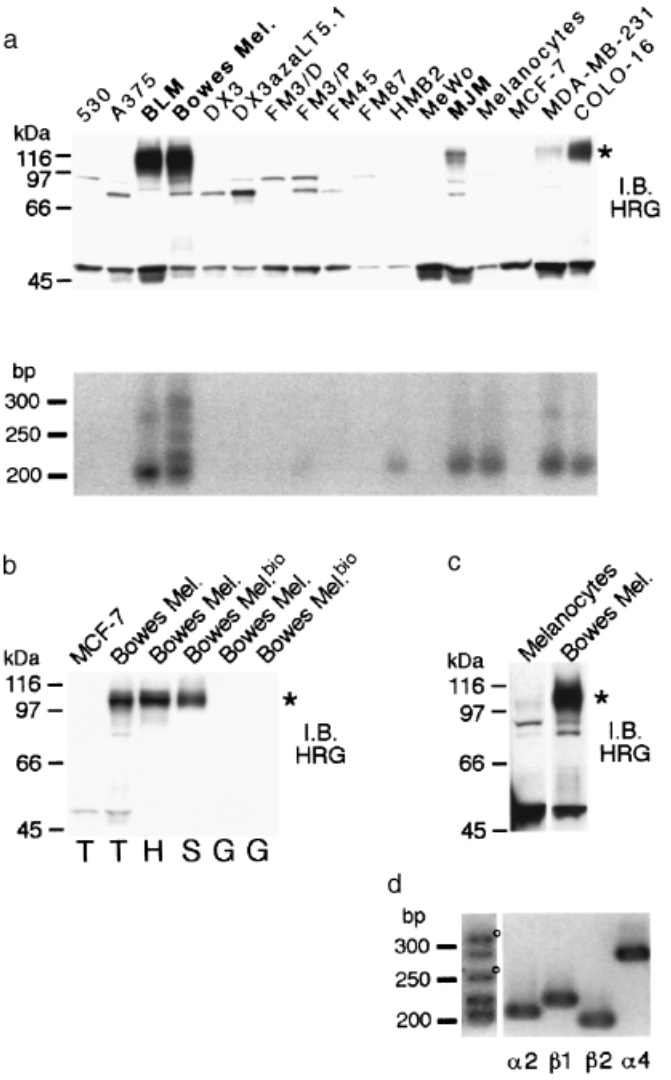


Figure 3. Presence of HRG protein and mRNA in melanocytes and melanoma cell lines. (*a, top panel; c*) HRG precursor expression was analyzed by immunoblotting (*I.B.*) of whole-cell lysates with an anti-HRG antibody. (*b*) Total lysates of MCF-7 and Bowes melanoma (*T*), a heparin precipitate (*H*), streptavidin precipitate (*S*), or control protein G–Sepharose precipitates of Bowes melanoma cells or biotinylated Bowes melanoma cells (*bio*) were analyzed by immunoblotting with an anti-HRG antibody. (*c*) Prolonged exposure after immunoblotting of total lysates of melanocytes and Bowes melanoma cells reveals a weak band in the former. *Asterisk,* the band of full-length HRG at 105 kDa. (*a, bottom panel*) RT-PCR analysis of HRG mRNA in the indicated cell lines using the primer panel indicated in the legend to **Fig 1**. (*d*) The *first lane* depicts HRG mRNA expression in Bowes melanoma as assessed by RT-PCR, using the primer panel indicated in the legend to **Fig 1**. *Lanes 2–5,* PCR analyses of clones derived from Bowes melanoma, representing the indicated HRG isoforms. *Open circle,* bands corresponding to PCR products formed by cross-annealing of two related isoforms and thus considered as aspecific.

both the exon leading to the α isoforms and the exons leading to the β_1 isoform (**Figs 1, 3*D***). Two bands (indicated with an *open circle*) did not correspond to a specific isoform because they were the result from cross-annealing of PCR products coming from β1–β2 or α2–β2 isoforms, presumably resulting in an imperfect double strand with slower migration on agarose gel (**Fig 3*D***).

Melanoma cells release functionally active HRG in the culture medium Following cleavage in the juxtamembrane

extracellular region, HRG are released into the culture medium as 40- to 45-kDa proteins, depending on the isoform and glycosylation level (Holmes *et al*, 1992; Lu *et al*, 1995a, b). Western blotting of 50 × concentrated CM, using an antibody directed against the HRG extracellular domain, revealed the presence of a broad band at the expected molecular weight (**Fig 4*A***, *lane 1*). This band was absent upon depletion of the concentrated CM from heparin-binding factors (**Fig 4*A***, *lane 2*).

To test whether the released HRG was functional, we verified whether CM from the melanoma cell lines was capable of activating HERs in MCF-7 cells. Prominent tyrosine phosphorylation of a 185-kDa protein was evident upon treatment with the positive controls recombinant HRG-β1 (rHRG-β1) and COLO-16 CM and was further restricted to CM from HRG-positive melanoma cell lines (**Fig 4*B***). This phosphorylation could be blocked by pretreating the MCF-7 cells for 30 min with the HER-specific irreversible inhibitor PD168393 (Fry *et al*, 1998) or by adding heparin to the CM (**Fig 4*C,D***). Heparin treatment did not interfere as such with the capability of HERs in MCF-7 cells to become activated, because the combination with rHRG-β1 (lacking a heparin-binding domain) still resulted in full phosphorylation of HERs in these cells (data not shown). To quantify the phosphorylating capacity of the Bowes melanoma CM, we made a comparison with the phosphorylation of MCF-7 cells that had been treated with different concentrations of rHRG-β1. As also shown by others (Aguilar and Slamon, 2001), phosphorylation of a 185-kDa protein could readily be detected using 0.5 ng per mL rHRG-β1 (**Fig 4*E***). Treatment with CM of Bowes melanoma cells resulted in a phosphorylation at 185-kDa equivalent to ±5 ng per mL (700 pM) rHRG-β1, correlating with a concentration of ±100 pM 45-kDa HRG in the CM. Also the kinetics of this phosphorylation were similar, with phosphorylation occurring already after 1 min of treatment, suggesting a similar mechanism of direct receptor activation (**Fig 4*F***).

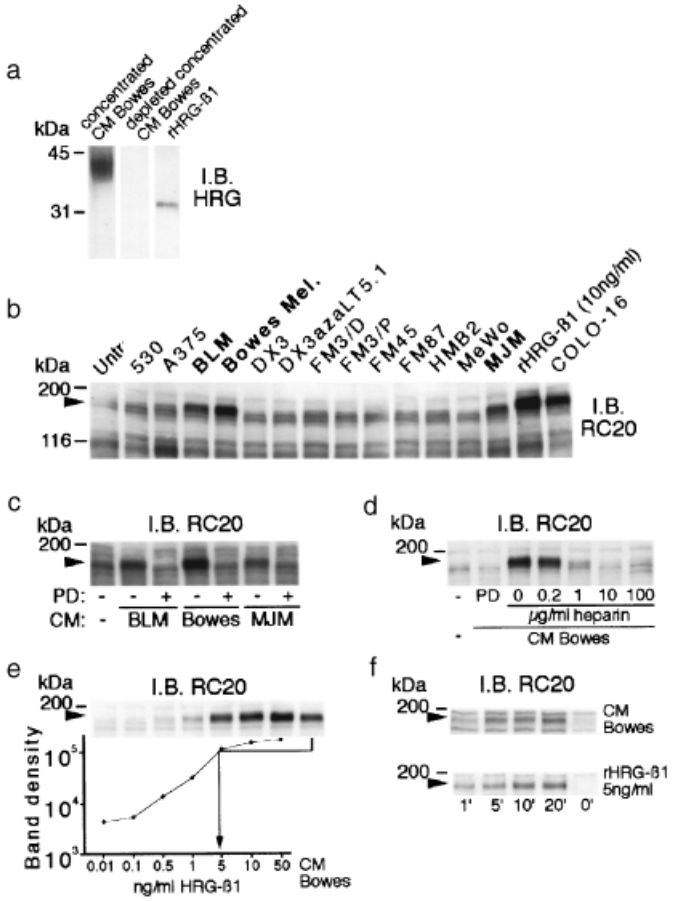


Figure 4. Melanoma cells release receptor activating HRG in the medium. (*a*) Immunoblotting (*I.B.*), using an anti-HRG antibody directed against the EGF-like domain, of concentrated CM of Bowes melanoma, before (*lane 1*) and after (*lane 2*) depletion of heparin-binding factors reveals the presence of a 45-kDa protein only in *lane 1.* Full-length recombinant HRG-β1 (*rHRG-β1*), produced in *Escherichia coli* (*lane 3*) migrates at 33 kDa owing to differences in glycosylation. (*b–e*) Analysis of tyrosine-phosphorylated proteins in serum-starved MCF-7 cells. (*b*) Cells treated for 30 min with serum-free medium (*Untr*), with rHRG-β1, or with CM from the indicated cell lines. (*c*) Cells pretreated or not for 30 min with 2 μM PD168393 (*PD*) and treated for an additional 30 min with serum-free medium or CM from the indicated cell lines. (*d*) Cells pretreated or not for 30 min with 2 μM PD168393 (*PD*) and treated with serum-free medium or with CM of Bowes melanoma to which heparin was added in the indicated concentrations. (*e*) Quantification of tyrosine phosphorylation, induced by treating MCF-7 cells with Bowes melanoma CM or by treating these cells with increasing concentrations of rHRG-β1. The *arrow* indicates that, by extrapolation, the phosphorylating capacity of Bowes melanoma CM is equivalent to that of ± 5 ng per mL rHRG-β1. (*f*) MCF-7 cells treated with 5 ng per mL rHRG-β1 or CM Bowes melanoma for the indicated periods of time. *Arrowheads,* ±185-kDa tyrosine-phosphorylated bands.

One of the well-described biologic effects of HRG is the rapid induction of spreading/scattering of epithelial islands (Spencer *et al*, 2000), which led us to test the effect of the CM of the HRG-positive melanoma cell lines in this assay. We found that a 2-h treatment of serum-starved MCF-7 islands with melanoma cell line CM resulted in a disruption of epithelial islands similar to that of treatment with rHRG-β1. This scattering was blocked by pretreating the cells for 30 min with PD168393 (**Fig 5*A***).

Based on the fact that HRG contains an extracellular heparin-binding domain, we performed precipitations using heparin beads on 50 × concentrated CM. Three consecutive precipitations completely abolished the ability of the CM to induce phosphorylation (**Fig 5*B***, *lane 4*) or spreading/scattering of epithelial MCF-7 islands (**Fig 5*C***). In contrast, after eluting the heparin-binding factors from the heparin beads, desalting, and dilution of these factors in serum-free medium, used for treating MCF-7 cells, the phosphorylation of a 185-kDa band (**Fig 5*B***, *lane 3*) as well as the induction of spreading/scattering (**Fig 5*C***) were evident. Both effects could be blocked by pretreating the cells for 30 min with PD168393 (**Fig 5*C,B***, *lane 6*).

An autocrine loop in Bowes melanoma cells leads to constitutive HER phosphorylation, MAPK activation, and increased growth Total lysates from different melanoma cell lines were immunostained for tyrosine-phosphorylated proteins. This revealed the presence of a highly phosphorylated protein at 185 kDa, possibly reflecting activated HER2 and HER3, in Bowes melanoma cells, but not in the other melanoma cell lines tested (**Fig 6*A***). This band was also found in the HRG-positive COLO-16 cells, but not in the weaker HRG-positive MDA-MB-231 cells. By precipitating HER2 and HER3 from Bowes melanoma cells and staining for tyrosine phosphorylated proteins, we could show constitutive phosphorylation of HER2 and HER3 (**Fig 6*B***, *middle panel*). Treating the cells for 30 min with PD168393 resulted in a complete block of this phosphorylation (**Fig 6*B***, *left and middle panels*). This was not due to alterations in receptor levels, as immunostaining for HER2 and HER3 showed no differences between untreated cells and cells treated with PD168393 (**Fig 6*B***, *right panel*). In line with this, when tested in a 5-day growth assay, PD168393 gave a significant ($p < 0.01$) and concentration-dependent growth inhibition of Bowes melanoma cells (**Fig 6*C***). This effect was not due to general cytotoxicity because virtually no growth inhibition was seen of MCF-7 cells (HER2/3-positive and HRG-negative) or BLM cells (HER2/HRG-positive and HER3-negative).

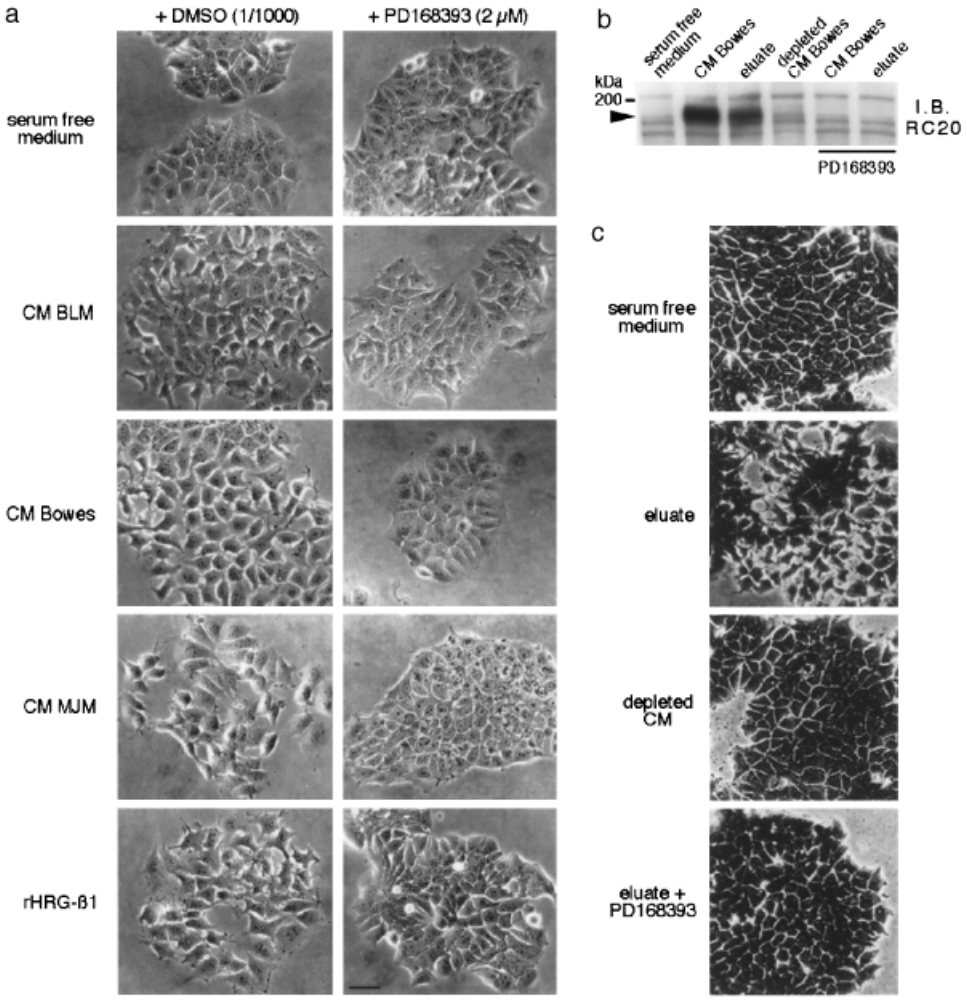


Figure 5. Scattering of MCF-7 epithelial islands by rHRG-β1 and by CM of HRG-positive cell lines. Serum-starved MCF-7 cells pretreated or not for 30 min with 2 μM PD168393, followed by an additional 30-min treatment and preparation of lysates (*b*) or followed by additional 2-h treatments before taking pictures of living cultures (*a*) or crystal-violet-fixed cultures (*c*). (*b*) Antiphosphotyrosine immunoblotting (*I.B.*) of whole-cell lysates of MCF-7 cells that were treated for 30 min with serum-free medium, CM of Bowes melanoma (*CM Bowes*), the heparin-binding fraction from CM Bowes (*eluate*) or CM Bowes depleted from heparin-binding factors. *Arrowhead,* ± 185-kDa phosphorylated band. (*c*) Indicated conditions as in (*b*). *Bars,* 50 μm.

Constitutive receptor activation in Bowes melanoma cells could be inhibited not only by directly blocking its kinase activity, but also the interference with ligand binding resulted in this effect. This was evident from the rapid, concentration-dependent inhibition of HER phosphorylation and the consequent growth inhibition seen upon treatment with heparin (**Fig 6*D,E***). The MAPK pathway is a major pathway implicated in uncontrolled growth of melanomas (Govindarajan *et al*, 2003; Satyamoorthy *et al*, 2003). Because it is also a well known signaling pathway activated by HRG (Pinkas-Kramarski *et al*, 1998), we checked its activation status in Bowes melanoma cells by western blotting using a phospho-MAPK-specific antibody. Constitutive HER phosphorylation of Bowes melanoma cells was accompanied by a constitutively active MAPK pathway (**Fig 6*F***, *lane 1*). Blocking HER phosphorylation with PD168393 rapidly led to a block of MAPK activation (**Fig 6*F***, *lanes 2,3*), showing that continuous HER activation is the main cause of the constitutively activated MAPK pathway in these cells. The importance of the continuous activation of this pathway for the growth of Bowes melanoma cells was shown in experiments in which we used PD98059, a MAPK inhibitor. Bowes melanoma cells were particularly sensitive to this inhibitor and showed significant growth inhibition at concentrations that did not have any effect on growth of control BLM or MCF-7 cells (**Fig 6*G***). In conclusion, constitutive HER activation by autocrine HRG supports growth of Bowes melanoma cells via continuous MAPK activation.

Exogenous HRG stimulates growth of melanoma cells and melanocytes but does not protect melanocytes against apoptosis Melanocytes depend for their survival *in vitro* strongly upon the addition of extracellular stimuli. A prominent growth factor promoting growth and survival of these cells is bFGF (Halaban, 2000). To test whether HRG could have similar effects, we treated melanocytes with different concentrations of rHRG-β1, bFGF, or the combination of both. As is evident from **Fig 7*A***, rHRG-β1 stimulated HER phosphorylation of melanocytes and a variety of melanoma cell lines. rHRG-β1 concentration-dependently stimulated growth of melanocytes and could even provide an additive stimulus over bFGF (**Fig 7*B***). A significant growth stimulation was also seen when, e.g., MCF-7, MeWo, and A375 cells were treated with rHRG-β1 (data not shown). Because bFGF is also a potent antiapoptotic factor for melanocytes (Alanko *et al*, 1999), we next tested whether HRG might have a similar effect. Upon seeding of melanocytes on a collagen gel, these cells undergo apoptosis, round up, and become annexin V-positive owing to the exposure of phosphatidylserine at the outer surface of the cells. This can be inhibited by adding bFGF to the medium (Alanko *et al*, 1999) (**Fig 7*C,D***). Although a small decrease in the percentage of apoptotic cells was reproducibly seen upon treatment with rHRG-β1, this effect was negligible compared to the antiapoptotic effect of bFGF (**Fig 7*C,D***). Overall, the results from these assays show that HRG potently stimulates growth of melanoma cells and melanocytes, but does not protect melanocytes against collagen-induced apoptosis.

Defective HRG/HER system in various melanoma cell lines As shown in **Fig 8*A***, phosphorylation in Bowes melanoma cells was already maximal, because treatment with

rHRG-β1 did not result in an increase of phosphorylation. Consistent with this, no additional growth stimulation could be seen upon treatment with rHRG-β1 (data not shown). The observation that the HRG-positive BLM cells lack constitutive receptor activation (**Fig 6*A***) and cannot be activated upon addition of rHRG-β1 (**Fig 8*A***) is in agreement with the absence of the HRG-receptor HER3 (**Fig 2*A***) in these cells. The lack of HER3 also accounts for the unresponsiveness of FM45 cells to rHRG-β1 (**Fig 8*A***). MJM cells seem to have a defective HER-system as well: although HER2 and HER3 were present (**Fig 2*A***), treatment with rHRG-β1 led only to a minor phosphorylation, compared with treated MCF-7 cells (**Fig 8*A***). This minor phosphorylation was due to a small increase in phosphorylation of HER3, but not of HER2 (**Fig 8*B***, *lane 2*). To check whether HER2 can be activated in MJM cells, we treated them for 10 min with pervanadate, a phosphatase inhibitor. This led to phosphorylation of multiple proteins, including HER2 and HER3 (**Fig 8*B***, *lane 3*). Cotreatment with rHRG-β1 and pervanadate led to an additional increase in phosphorylation of only HER3 (**Fig 8*B***, *lane 4*), compared to treatment with pervanadate only. To verify whether mutations in HER2 could be responsible for the lack of activation in response to signaling from outside the cell, we sequenced all exons of the *HER2* gene. Apart from described polymorphisms in the sequences encoding the transmembrane domain (Ile^{655} to Val^{655}) (Ehsani *et al*, 1993) and the C-terminal tail (Pro to Ala), no mutations were found. Furthermore, biotinylation revealed that full-length HER2 was present at the plasma membrane of MJM cells (data not shown). So, despite the lack of mutations of HER2 and its localization at the plasma membrane in MJM cells, this receptor lacks the potential to become activated via stimulation with ligands.

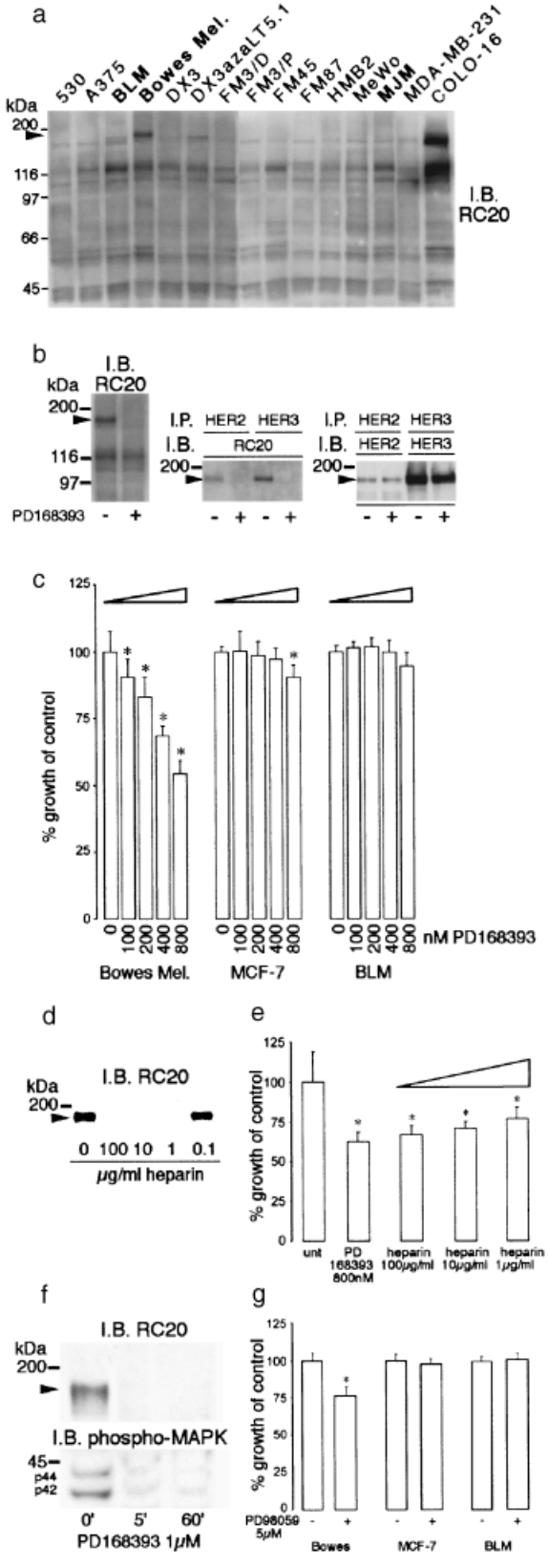


Figure 6. Correlation between constitutive HER activation in Bowes melanoma, continuous MAPK activation, and increased growth. (*a*) Whole lysates from 48-h serum-starved cell lines analyzed by immunoblotting (*I.B.*) using an antiphosphotyrosine antibody. (*b,d*) Immunoblotting of tyrosine-phosphorylated proteins in serum-starved Bowes melanoma cells, treated or not with 2 μM PD168393 for 30 min (*b*) or with different concentrations of heparin for 10 min before making cell lysates (*d*). (*b*) The *left panel* indicates whole-cell lysates. *Middle and right panels,* equal amounts of protein were immunoprecipitated (*I.p.*) using anti-HER2 or anti-HER3 antibodies, before immunoblotting with anti-phosphotyrosine, anti-HER2, or anti-HER-3 antibodies. (*f*) Bowes melanoma cells treated with 1 μM PD168393 for different periods of time. Whole-cell lysates were analyzed by immunoblotting using antiphosphotyrosine (*top panel*) or antiphospho-MAPK antibodies (*bottom panel*). *Arrowheads,* ±185-kDa phosphorylated bands. (*c,e,g*) Growth, relative to vehicle-treated cells, as measured by sulforhodamine B assay. Cells were treated for 5 days with the indicated concentrations of PD168393 (*c,e*) or heparin (*e*) or with 5 μM PD98059 (*g*). *Asterisks,* differs significantly ($p < 0.01$) from controls.

DISCUSSION

In this report, we investigated the expression and function of the HRG/HER ligand–receptor system in 13 melanoma cell lines, compared to normal melanocytes. HER2 and HER3 were found to be the main members of the EGFR family expressed in these cells. Nevertheless, these receptors were not overexpressed, which is consistent with the analysis of both nevi and melanoma tumor material by others (Natali *et al*, 1994; Korabiowska *et al*, 1996; Persons *et al*, 2000; Fink-Puches *et al*, 2001). Similar amounts of HER3 protein were present in melanocytes and in 11 of the 13 cell lines. The fact that two melanoma cell lines (BLM and FM45) showed only low HER3 mRNA levels and even no detectable HER3 protein is a first example of how the HRG/HER system may be deregulated in melanoma (**Fig 9**). Loss of HER3 may imply that transformed melanocytes no longer depend on HRG, normally provided by the surrounding keratinocytes (Schelfhout *et al*, 2000), for their survival. Downregulation or loss of other melanocytic RTK (e.g., c-Kit, protein-tyrosine kinase 4, ephrin receptor EphA4) during melanoma progression has been

described before (Easty and Bennett, 2000). Their loss may uncouple the melanocytes from certain physiologic regulatory mechanisms or may indicate an independence from the growth factor system involved. In the latter case, other systems (autocrine/paracrine) and/or activating mutations of other (intracellular) proteins may have substituted for the loss. In this context, it is noteworthy that we found a mutated N-*ras* allele in BLM, and also in MJM cells (see further), resulting in a constitutively active N-ras (data not shown), whereas FM45 cells have a mutation in the tumor suppressor PTEN (Guldberg *et al*, 1997), resulting in

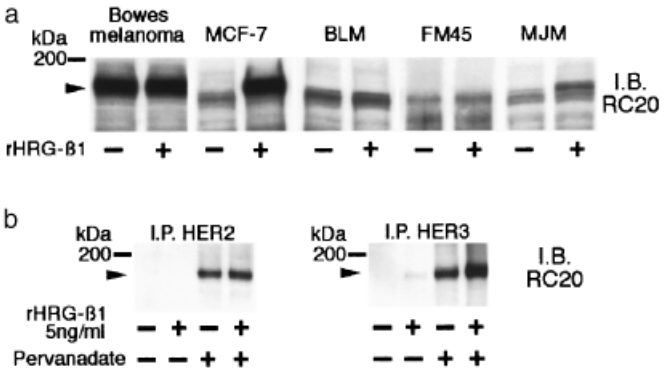
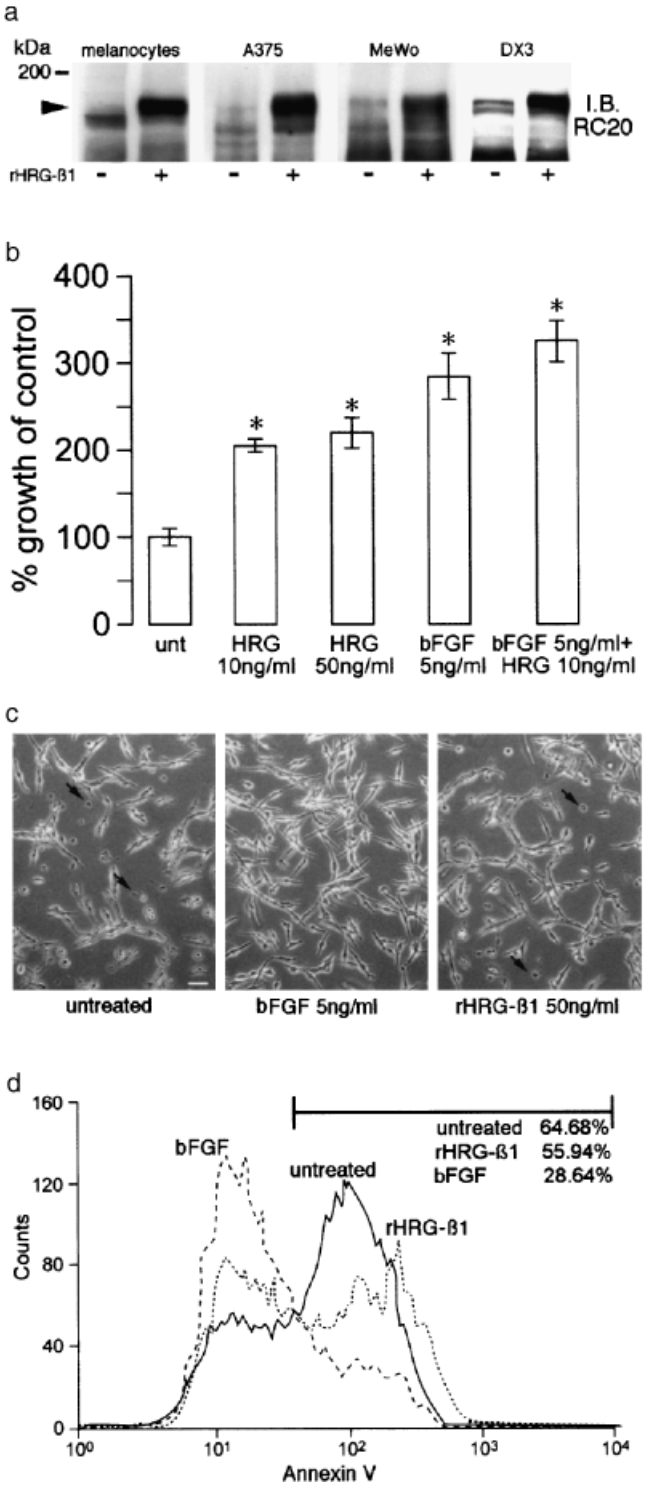


Figure 8. Deregulated HRG/HER system in several melanoma cell lines. (*a*) Serum-starved cells, treated with rHRG-β1 or not before the preparation of whole-cell lysates and analysis by immunoblotting (*I.B.*) using an antiphosphotyrosine antibody. (*b*) Equal amounts of protein from cell lysates of MJM cells that had been treated for 10 min with rHRG-β1 and/or pervanadate were subjected to immunoprecipitation (*I.p.*) with anti-HER2 or anti-HER3 antibodies before immunoblotting using an antiphosphotyrosine antibody. *Arrowhead,* ±185-kDa phosphorylated band.



constitutive phosphoinositide 3-kinase signaling. We have evidence (unpublished data) that the absence of HER3 in BLM and FM45 cells is accompanied by the loss of microphtalmia transcription factor M, a melanocyte-specific transcription factor necessary for melanocyte development. Interestingly, expression of both HER3 and microphtalmia transcription factor M has been described to be under the control of SOX-10, a transcription factor necessary for melanocyte development, thus making this protein a putative regulatory candidate (Verastegui *et al*, 2000; Britsch *et al*, 2001).

The MJM cell line represents a second example of a deregulated HRG/HER system in melanoma (**Fig 9**). Although it expresses HRG, HER2, and HER3, surprisingly, it cannot use the secreted HRG in an autocrine loop. Following treatment of MJM cells with exogenous HRG, HER2 is not activated at all, whereas HER3 becomes only weakly phosphorylated. The fact that a minute phosphorylation of HER3 still occurs is surprising, because HER3 lacks catalytic activity and needs HER2 for its phosphorylation in the absence of other HERs. We cannot exclude that HER1 or HER4, whose levels were below the detection limit of our western blotting experiments, account for this effect. We can exclude the possible (lack of) regulatory action of heparan sulfate proteoglycans to be responsible for the impaired response, because the used rHRG-β1 only consists of the EGF-like domain. Also the interference by circulating soluble HER2

Figure 7. Receptor-activating and growth-promoting, but no anti-apoptotic effects of exogenous HRG on melanoma cells and melanocytes. (*a*) Immunoblotting (*I.B.*), using an antiphosphotyrosine antibody, of lysates from serum-starved cells, treated or not with rHRG-β1. *Arrowhead,* ±185-kDa phosphorylated band. (*b*) Growth, relative to untreated cells, as measured by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Melanocytes were treated for 5 days with the indicated concentrations of growth factors. *Asterisks,* differs significantly ($p < 0.01$) from control. (*c*) Phase contrast photographs of melanocytes cultured on a collagen gel for 4 days with the treatments indicated. *Arrows,* rounded cells in melanocyte cultures that were left untreated or were treated with rHRG-β1. These rounded cells were not found in bFGF-treated cultures. *Bar,* 50 μm (*d*) Histogram, showing the profile of annexin V positivity in melanocytes that were cultured on a collagen gel in the absence of added growth factors (*solid line*) or in the presence of 5 ng per mL bFGF (*broken line*) or 50 ng per mL rHRG-β1 (*dotted line*). The percentage of annexin V-positive cells is indicated.

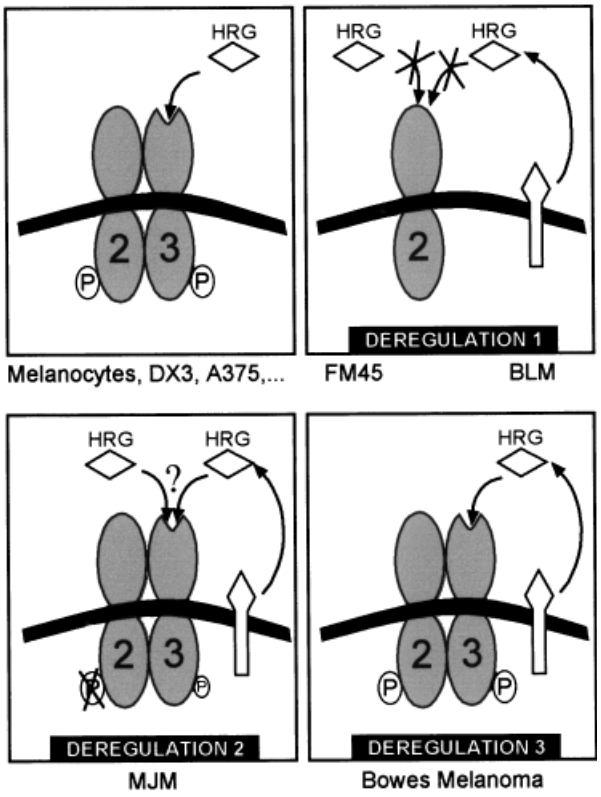


Figure 9. Schematic representation of the several deregulations of the HRG/HER system found in human melanoma cells. In melanocytes and the majority of melanoma cell lines, HERs can be activated by exogenous HRG. The absence of HER3 in BLM and FM45 cells or the presence of functionally inactive HER2 in MJM hampers HRG responsiveness. Bowes melanoma cells show constitutive HER activation, owing to the presence of an autocrine loop.

or HER3 ectodomains (Doherty *et al*, 1999; Aigner *et al*, 2001; Lee *et al*, 2001; Justman and Clinton, 2002) is unlikely, because CM from the HRG-positive MJM cells still induced activation of HERs in MCF-7 cells. Biotinylation experiments suggested a transmembrane localization of HER2 in MJM cells, whereas analysis of functional sequences failed to show mutations. Nevertheless, sequencing did reveal two polymorphisms, one of which (Ile to Val at position 655 in the HER2 transmembrane domain) was predicted to favor the formation of stable HER2 homodimers (Fleishman *et al*, 2002). Although it still needs to be shown whether the proposed model also holds for HER2 activation by heterodimerization (following ligand binding), it would mean that HER2 in MJM cells would be more easily activated than in, e.g., Bowes melanoma cells, which is in contrast to our findings. It thus seems unlikely that this polymorphism may provide an explanation for the observed lack of HER2 activation in MJM cells treated with HRG. A possible role for the other polymorphism we identified in MJM cells has not been established yet. Remaining explanations for the lack of HER2 activation following stimulation with HRG are the interference with ligand binding by sterical hindrance, the constitutive association or action of intracellular negative regulatory proteins or receptor mislocalization (although transmembrane).

Here, we have shown by western blotting, RT-PCR, cloning, and using functional assays the presence and function of HRG as new growth factors produced by human melanoma cells. In addition to three known HRG isoforms, we could identify a new isoform, designated α_4. This isoform combines the sequences that normally lead to either α- or β-isoforms. A similar combination was already described for the α_3-isoform, which differs from the α_4-isoform because the latter contains the coding sequence for the transmembrane domain (Wen *et al*, 1994) (**Fig 1**). Nevertheless, the resulting protein is the same, because this α–β combination leads to a frameshift, resulting in the generation of a stop codon upstream of the sequence encoding the transmembrane domain. This truncated protein is most likely cytosolic because the transmembrane domain functions as a signal peptide (Burgess *et al*, 1995). The presence of HRG in melanomas fits with the neuroectodermal origin of melanocytes and the fact that HRG are molecules typically expressed in neuroectodermal tissues (Marchionni *et al*, 1993; Meyer and Birchmeier, 1995). Although melanocytes showed HRG expression at the mRNA level, HRG could barely be detected at the protein level, suggesting the presence of (post)translational negative regulatory mechanisms in these cells. Activating mutations in H-*ras* have been shown to result in upregulation of HRG in mammary epithelial cells (Mincione *et al*, 1996). Although two of the HRG-producing cell lines (BLM and MJM) have an activating mutation in N-*ras*, transient transfections using dominant-negative and constitutively active N-ras constructs learned that this was unlikely to be the underlying cause of the increased HRG expression (data not shown). Thus, the molecular basis for the high HRG expression in some melanoma cell lines is not clear, yet. Although exogenous HRG did not exert a significant antiapoptotic effect, it potently stimulated growth of cultured melanocytes and melanoma cells and could even provide an additive growth stimulation over bFGF. Upregulation of HRG expression in melanomas may result in the generation of an autocrine loop and in the independence from HRG normally provided by the keratinocytes (Schelfhout *et al*, 2000). This decreased dependence from paracrine growth factors is one of the hallmarks of melanoma progression (Lázár-Molnár *et al*, 2000). Our data clearly show that in the Bowes melanoma cell line, in the absence of receptor overexpression, HER2 and HER3 are permanently activated, leading to continuous MAPK activation and stimulation of growth. This activation is due to continuous ligand–receptor interactions and not to, e.g., activating mutations. Arguments hereto are that the phosphorylation could be abolished by adding heparin to the culture medium and that refreshing of the culture medium led to a transient, gradual decrease in receptor phosphorylation, followed by a gradual recovery to the initial levels (data not shown). Thus, the Bowes melanoma cell line, with its autocrine loop, represents a third example of how the HRG/HER system may be deregulated in melanoma (**Fig 9**). Expression of the HRG/HER system was also described in various other types of cancers (e.g., breast, lung, endometrium, thyroid, head and neck, colon, ovarium) (Ethier *et al*, 1996; Fernandes *et al*, 1999; Srinivasan *et al*, 1999; Fluge *et al*, 2000; O-Charoenrat *et al*, 2000; Gilmour *et al*, 2002; Venkateswarlu *et al*, 2002). Although in only some of these studies constitutive receptor activation, owing to an autocrine loop, was looked at, it may play a role in the other cases as well, rendering it a possible target for future therapies. In line with this is the increased attention that is being given toward receptor activation status in certain cancers, rather than only taking into account receptor levels as a criterium of malignancy (Thor *et al*, 2000).

Overall, it is striking that two of the three HRG-positive cell lines, BLM and MJM, cannot use the secreted HRG in an autocrine loop, because of the absence of HER3 or because of an impaired HER2 activation, respectively. Still, in a physiologic situation, the HRG secreted by such cells may have prominent effects on the surrounding cells, directly or indirectly contributing to malignant progression. Direct effects may include an increased motility of the surrounding keratinocytes (Schelfhout *et al*, 2002), possibly rendering the environment in which the melanocytes reside less tight. Indirect effects may be the stimulation of angiogenesis owing to a HRG-mediated upregulation of vascular endothelial growth factor or increased expression of other growth factors by the target cells (O-Charoenrat *et al*, 2000; Talukder *et al*, 2000; Yen *et al*, 2000; Ruiter *et al*, 2002).

In summary, we have shown the presence of HRG, including one new isoform, as new factors produced and secreted by human melanoma cell lines. The HRG/HER system is functional in melanocytes and in the majority of melanoma cell lines, leading to growth stimulation. Nevertheless, multiple deregulations in this growth factor system may release the melanocytes from their natural dependence on keratinocyte-derived factors and thus represent a step toward melanoma progression. Lack of stimulation by HRG in some melanoma cell lines is due to the loss of expression of HER3 protein or to a severely impaired HER2 activation. In contrast, the aberrant expression and secretion of HRG by melanoma cells may serve as an autocrine and/or paracrine signal, promoting cell growth and/or migration. These distinct types of deregulation of the HRG/HER system may contribute to the malignant phenotype of melanoma cells. In the future, it will be important to verify whether these deregulations are present in tumor samples of melanoma patients and may become a therapeutic target for this disease with ever increasing incidence.

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