Tumor and Stem Cell Biology

Constitutive HER2 Signaling Promotes Breast Cancer Metastasis through Cellular Senescence

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Abstract

Senescence, a terminal cell proliferation arrest, can be triggered by oncogenes. Oncogene-induced senescence is classically considered a tumor defense barrier. However, several findings show that, under certain circumstances, senescent cells may favor tumor progression because of their secretory phenotype. Here, we show that the expression in different breast epithelial cell lines of p95HER2, a constitutively active fragment of the tyrosine kinase receptor HER2, results in either increased proliferation or senescence. In senescent cells, p95HER2 elicits a secretome enriched in proteases, cytokines, and growth factors. This secretory phenotype is not a mere consequence of the senescence status and requires continuous HER2 signaling to be maintained. Underscoring the functional relevance of the p95HER2-induced senescence secretome, we show that p95HER2-induced senescent cells promote metastasis in vivo in a non-cell-autonomous manner.

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Introduction

Senescence, an irreversible cell proliferation arrest, can be triggered by an excessive number of cell divisions or a variety of stressors, including oncogenes. Oncogene-induced senescence (OIS) constitutes an antitumor barrier that impedes the expansion of early neoplastic cells before they become malignant (1, 2). However, senescent cells remain metabolically active and, through a robust secretory machinery (3), release a wealth of factors, collectively termed senescence-associated secretory phenotype (SASP) or senescence messaging secretome (SMS) (4, 5). This senescence secretome includes components necessary to establish and maintain the senescence program (5) and, in addition, chemotactic factors that mediate the clearance of senescent cells in vivo by attracting cellular components of the immune system belonging both to the innate and to the adaptive immune response (6–8). However, the frequent presence of protumorigenic factors in the senescence secretome has led several authors to propose that, under certain circumstances, OIS may contribute to tumor progression in a cell nonautonomous manner (4, 5).

The receptor tyrosine kinase HER2 is a prototypic proto-oncogene overexpressed in approximately 20% of breast cancers. HER2-positive tumors constitute a group of breast cancers with specific biologic features and therapeutic options (9). The expression of neu, an oncogenic mutant form of HER2, leads to premature senescence (10); however, very little is known about the relevance of this observation in the progression and treatment of HER2-positive breast tumors.

A subgroup of HER2-positive breast cancers express a heterogeneous group of 80 to 115-kDa carboxy-terminal fragments of HER2 collectively known as HER2 CTFs or p95HER2 (11). Compared with tumors expressing only full-length HER2, p95HER2-positive tumors exhibit worse prognosis and a higher likelihood to metastasize (12, 13). One of the HER2 CTFs, the 100- to 115-kDa p95HER2 fragment (also known as 611-CTF), is a constitutively active form of HER2 because of its ability to form homodimers maintained by disulphide bonds (14). Here, we show that expression of 110- to 115-kDa p95HER2/611-CTF (hereafter referred to as p95HER2) can induce the onset of OIS in different breast cancer cells. Notably, p95HER2-induced senescent cells, likely due to their distinct secretory phenotype, increase the ability of proliferating breast cancer cells to metastatize.

Materials and Methods

Materials

Antibodies were from Dako (anti-Ki67), BD Biosciences (Rb), Cell Signaling (anti-P-HER2 (Y1221/1222), anti-Ras, anti-P-π53 (Ser15), Santa Cruz Biotechnology (anti-p21, anti-53BP1 and anti-p53), BioGenex [anti-HER2 (C11)], Amersham [anti-rabbit IgG and anti-mouse IgG, both horseradish peroxidase (HRP)-linked], Invitrogen [anti-mouse-Alexa 488, anti-mouse-
PE, anti-goat-Alexa 568, and Milipore (anti-gamma-H2AX). Lapatinib was kindly provided by GlaxoSmithKline.

Doxorubicin was from Sigma-Aldrich, MMP1, ANGPTL4 (RayBiotech), interleukin (IL)-11 (R&D), and IL-6 (eBiogenesis) ELISA kits were used for determination of the corresponding factors in conditioned media or serum according to the manufacturer's indications.

**Cell culture**

MCF7 Tet-Off/p95HER2, MCF7 Tet-Off/HER2, and T47D/p95HER2 cells were transfected as previously described (14).

p95HER2_MDA-MB-453 and p95HER2_MCF10A were obtained by retroviral transduction with p95HER2. p95HER2_MDA-MB-453 were maintained in L15 + GlutaMAX (Gibco) containing 10% FBS, 0.75 μg/mL puromycin (Sigma), and 1 μmol/L lapatinib (Tykerb, GlaxoSmithKline), whereas p95HER2_MCF10A were maintained in Dulbecco's Modified Eagle's Media (DMEM):F-12, 10% FBS, 4 mmol/L-l-glutamine, and 0.75 μg/mL puromycin.

MDA-MB-231/Luc were obtained by retroviral transduction as previously described (15).

**Western blot and confocal microscopy**

Western blot and confocal microscopy were carried out as previously described (14).

**Proliferation assay**

Proliferation was analyzed by cell counting. After trypsinization, viable cells determined by trypan blue dye exclusion were counted on a Neubauer chamber.

**WST1 assay**

The WST1 reagent was from Roche. A total of 5 × 10³ cells were seeded in 96-well plates and the assay was conducted following the manufacturer's indications.

**Metabolic labeling**

Approximately 3 × 10⁶ cells were metabolically labeled with 500 μCi/mL [³⁵S]Translabel for 45 minutes in cysteine and methionine-free medium and lysed. Cell lysates were normalized by the number of cells and analyzed on SDS-PAGE and fluorography.

**Determination of cell volume**

Cells were trypsinized, resuspended in complete medium, and cell diameter was determined by direct measuring in a Neubauer chamber. Cell volume was approximated to the one of a sphere as 4/3 × (π × cell radius²) and 5 representative fields with 10 to 15 cells were analyzed.

**Senescence-associated β-galactosidase activity**

Both cells and tissue slides were analyzed using senescence β-galactosidase staining kit (Cell Signaling Technology) following the manufacturer's indications.

**Cell irradiation**

Cells were trypsinized, resuspended in complete medium, and transferred to a 15-mL falcon tube. About 10 Gy γ-irradiation dose was applied at Radiotherapy Service of the Vall d’Hebron University Hospital (Barcelona, Spain) with a cobalt unit (Theraton 780-C. NCA) at a dose rate of 80 cGy/min and the total dose was 10 Gy in a single dose.

**ELISAs**

The conditioned media were collected, spun down at 200 × g for 5 minutes, and transferred into clean tubes. Mice sera were obtained by complete exsanguinations and subsequent centrifugation using heparinized material. Concentration of all factors was determined according to the manufacturer's instructions of each kit, normalized to cell number, and expressed as pg/mL/25,000 cells. All the experiments were carried out at least 3 times, and the results are represented as the means ± SD.

**Immunohistochemistry**

Tumor xenografts were removed, fixed overnight with 4% formalin, and then paraffin-embedded. Sequential 5-μm thick slices where then obtained, hematoxylin and eosin stained, and immunostained for Ki67, p21 (immunohistochemistry), and γ-H2AX (immuno-fluorescence).

**Xenografts**

Mice were maintained and treated in accordance with institutional guidelines of Vall d’Hebron University Hospital Care and Use Committee. p95HER2_MCF7 Tet-Off cells were injected into the right flanks of 6- to 8-week-old female BALB/c athymic mice purchased from Charles Rivers Laboratories. The expression of p95HERs was repressed by adding doxycycline to the drinking water until tumors were about 150 mm³. Then mice were randomized and treated with or without doxycycline (50 mg/kg/d). Tumor xenografts were measured with calipers every 3 days, and tumor volume was determined using the formula: (length × width²) / 2. At the end of the experiment, the animals were anesthetized with a 1.5% isoflurane-air mixture and were killed by cervical dislocation. Results are presented as mean ± SD of tumor volume.

Metastatic colonization was monitored by in vivo bioluminescence imaging using the IVIS-200 Imaging System from Xenogen as previously described (16).

**Transcriptomic analysis**

Expression analysis in both MCF7 was conducted using Affymetrix gene chips HG U133 2.0, as previously described (14).

**Proteomic analysis**

Cells expressing or not p95HER2 during 5 days were washed 5 times with serum-free medium and incubated for additional 48 hours in the absence of serum. The conditioned media were then collected, spun down at 200 × g for 5 minutes, transferred into clean tubes, filtered through a Nalgene 0.2-μm pore vacuum filter (Fisher #09-741-07), and concentrated using a 10 000 MWCO Millipore Amicon Ultra (Millipore #UFC901024) spinning down 15 mL at a time at 800 × g for 30 minutes until the final concentration was 1 mg/mL (~200- to 300-fold concentration). Protein concentration was determined with
a Bio-Rad protein assay (Bio-Rad, #500-0006). Subsequent sample preparation and proteomic analysis were conducted as previously described (17).

**Statistical analysis**

Data are presented as averages ± SD and were analyzed by the Student t test when comparing 2 groups or ANOVA when comparing more than 2 groups. Results were considered to be statistically significant at \( P < 0.05 \). All statistical analyses were conducted using the SPSS 12.0 Statistical Software (SPSS, Inc.).

**Results**

**Effect of p95HER2 expression in different breast epithelial cell lines**

In MCF10A, a nontransformed immortalized mammary epithelial cell line, the expression of p95HER2 accelerated cell proliferation (Supplementary Fig. S1A). In contrast, in MCF7, MDA-MB-453, or T47D, the expression of the HER2 fragment resulted in a marked proliferation arrest and increased levels of the senescence-associated \( \beta \)-galactosidase activity (SA-\( \beta \)-gal; Fig. 1A; Supplementary Fig. S1B and S1C), 2 phenotypes associated with OIS.

OIS is irreversible and it is characterized by the activation of the DNA damage response (DDR). The proliferation arrest was irreversible after 48 hours of p95HER2 expression (Fig. 1B), and it was accompanied by the upregulation of 2 markers of the activation of the DDR: \( \gamma \)-H2AX (the phosphorylated form of histone H2AX) and 53BP1 (tumor suppressor \( p53 \)-binding protein; Fig. 1C).

OIS is regulated by \( p53 \) and/or \( pRb \) pathways and results in an increased expression of cyclin-dependent kinase inhibitors (CDKI). The expression of p95HER2 resulted in the activation of both pathways and in the upregulation of the CDKI p21 (Fig. 1D). As a control, we showed that treatment with the DNA-damaging agent doxorubicin, which promotes senescence in MCF7 cells (18), led to comparable results (Fig. 1D).

Senescent cells remain metabolically active (1). We observed that the metabolic activity of MCF7 cells expressing p95HER2 was higher than that of proliferating nonexpressing cells as judged by the WST1 assay, which is frequently used to...
determine cell proliferation but, in reality, it measures dehydrogenase activity (Supplementary Fig. S2A). Furthermore, p95HER2 expression led to an increased rate of protein biosynthesis (Supplementary Fig. S2B). This enhanced metabolic activity is the likely cause of the remarkable hypertrophy experimented by p95HER2-expressing cells (Supplementary Fig. S2C and S2D).

Collectively, these results showed that expression of p95HER2 in different breast cancer cells leads to OIS.

**p95HER2-induced senescence secretome**

While expression of p95HER2 in MCF7 cells results in OIS (Fig. 1), the expression of full-length HER2 does not prevent the proliferation of the same cells (Supplementary Fig. S1D, see also Fig. 2A). The majority of genes regulated by p95HER2 are also regulated by full-length HER2. However, a group of genes is specifically regulated by the constitutively active HER2 fragment (14). Therefore, to identify components of the secretome specific of the senescence state, we focused in genes regulated by p95HER2 but not by HER2 (Fig. 2B; Supplementary Table S1). Of the 1,631 genes regulated by p95HER2 (log2FC > 0.9), 944 were also regulated by HER2, whereas 2 groups of 320 and 367 genes were more acutely up- or downregulated, respectively, by p95HER2 (Fig. 2B).

Nearly one fifth of the genes preferentially upregulated by p95HER2 encode for transmembrane proteins or secreted factors and therefore they could contribute to the secretome of p95HER2-induced senescent cells. To validate and extend this observation, we compared the secretome of p95HER2-induced senescent MCF7 cells with that of control MCF7 cells through label-free proteomics. We identified 361 proteins whose levels increased (log2FC > 0.9) in p95HER2-induced senescent cells (Supplementary Table S2). Fifty-five of the corresponding genes were transcriptionally upregulated in p95HER2-induced senescent cells (Fig. 2C; Supplementary Table S3). Many of these secreted factors, such as matrix metalloproteases 1 (MMP1), angiopoietin-like 4 ANGPTL4, IL-11, and IL-6, are well-characterized protumorigenic factors (19, 20). Analysis of the levels of these factors by ELISA confirmed their increased secretion by p95HER2-induced senescent cells and their absence in the media conditioned by cells expressing HER2 (see Fig. 3A).

The p95HER2-induced senescence secretome is regulated by HER2 signaling

The secretory phenotype is considered one of the hallmarks of premature senescence (21). Therefore, it could be speculated that the secretory phenotype of p95HER2-induced senescent cells is a consequence of the senescence status and not a consequence of the expression of p95HER2. To test this possibility, we used the HER2 tyrosine kinase inhibitor lapatinib. As expected, lapatinib did not revert the senescence phenotype (Supplementary Fig. S3); nevertheless, the inhibitor impaired the production of the factors analyzed (Fig. 3A), strongly...
suggesting that their efficient secretion requires continuous p95HER2 signaling. To confirm this conclusion, we induced cellular senescence by irradiation (Fig. 3B and C). Irradiation-induced senescent cells did not secrete detectable levels of any of the factors analyzed (Fig. 3D), but induction of p95HER2 in irradiation-induced senescent cells resulted in a secretory phenotype similar to that of p95HER2-induced senescent cells (Fig. 3C and D). As a further control, we showed that treatment with lapatinib blocked the secretion of MMP1, IL-11, and IL-6 and impaired the secretion ANGPTL4 (Fig. 3D). We concluded that, in addition to trigger senescence, the expression of active p95HER2 is required to maintain the p95HER2-induced senescence secretome. However, the maintenance of the senescence state and the composition of the senescence secretome are regulated independently.

**Dynamics of the p95HER2-induced senescence secretome in vitro and in vivo**

Characterization of the dynamics of the senescence secretome induced by p95HER2 in MCF7 cells showed that in vitro senescent cells continue secreting high levels of IL-6, IL-11, MMP1, and ANGPTL4 for at least 1 month (Fig. 4A and B). This result indicates that p95HER2-induced senescent cells could constitute a long-lasting reservoir of protumorigenic factors in vivo. To test this hypothesis, we injected MCF7 Tet-Off p95HER2 cells into nude mice and when the tumors reached about 150 mm³, we removed doxycycline from the drinking water of the animals to allow the expression of p95HER2 (Fig. 3C). The subsequent analysis of xenograft samples showed the efficient onset of senescence in vivo after about 21 days of expression of p95HER2 as judged by the decrease of the cell proliferation marker Ki67, increase in the percentage of cells positive for p21, γ-H2AX, and SA-β-gal (Fig. 3D; Supplementary Fig. S4A). Consistently, the xenografts expressing p95HER2 grew for about 30 days, probably because of the increase in cell size, and then stabilized (Fig. 3C). Furthermore, cells obtained from xenografts expressing p95HER2 displayed the typical morphology of senescent cells (Supplementary Fig. S4B and S4C). A time course determination of the plasma levels of ANGPTL4 and IL-11 in mice carrying senescent cells

![Figure 3. Effect of inhibition of HER2 signaling on the p95HER2-induced senescence secretome. A, results of ELISAs to determine the concentration of the indicated factors in the conditioned media of MCF7 Tet-off/p95HER2 and MCF7 Tet-off/HER2 treated with or without doxycycline and lapatinib (Lap) as indicated. P values were obtained by 2-tailed Student \( t \) test. *, \( P < 0.05 \); **, \( P < 0.001 \). nd, not detectable. B, MCF7 Tet-off/p95HER2 treated with doxycycline were irradiated with 10 Gy. One week after irradiation, control or irradiated cells were fixed and stained for \( \beta \)-galactosidase (\( \beta \)-gal) activity. Representative phase contrast microscopy images are shown. C, schematic drawing showing the protocol used; see text for details. D, results of ELISAs to determine the concentration of the indicated factors in the conditioned media of irradiated MCF7 Tet-off/p95HER2 treated with or without doxycycline and or lapatinib.](image-url)
expressing p95HER2 showed that the senescence secretome is also displayed in vivo during long periods of time (Fig. 4E).

These results show that the p95HER2-induced senescent cells are long lived in vitro and in vivo and that they continuously secrete protumorigenic factors.

**p95HER2-induced senescent cells favor metastasis cell nonautonomously**

MDA-MB-231, a cell line established from the pleural fluid of a patient with advanced metastatic breast cancer, is a widely used experimental model of breast cancer metastasis. Injection of MDA-MB-231 cells carrying luciferase as reporter into the hearts of nude mice results in colonization of bones, brain, or lungs that can be monitored in vivo by bioluminescence imaging (22).

The increase in plasma levels of different prometastatic factors (Fig. 4E) suggests that the presence of p95HER2-induced senescent cells in the primary tumor contributes to metastasis in a systemic manner. To this aim, we injected MDA-MB-231/Luc cells intracardially in mice carrying subcutaneous MCF7 Tet-off/p95HER2 xenografts and treated them with or without doxycycline. Although in both conditions, 100% of the mice developed metastases, the metastatic cells that colonized target organs in mice carrying p95HER2-
induced senescent cells gave rise to bigger metastasis, as measured by total photon flux emission (Fig. 5A and B). This result shows that the p95HER2-induced senescent cells, likely through the secretion of prometastatic factors, act in a systemic fashion increasing the metastatic growth of cells that have reached the target organs. As a control, we ruled out that the observed results were due to the effect of the removal of doxycycline on MDA-MB-231/Luc cells (Supplementary Fig. S5).

Many of the factors secreted by senescent cells are likely to exert their functions locally. For example, cell surface and secreted proteases tend to cleave extracellular components in close proximity to the producing cell. Therefore, we analyzed the metastatic behavior of MDA-MB-231/Luc cells co-injected orthotopically with MCF7 Tet-off/p95HER2 cells. The presence in the primary tumor of p95HER2-induced senescent cells did significantly increase the metastatic ability of MDA-MB-231 cells (Fig. 5C and D). This result was likely not due only to the differences in the growth rate of the primary tumor, as shown by tumor volume and luminescence (Supplementary Fig. S6). Therefore, p95HER2-induced senescent cells prime proliferating breast tumor cells for metastasis.

Discussion

The main argument supporting a positive contribution of senescence to tumor progression is the existence of the senescence secretome, which is enriched in protumorigenic cytokines, growth factors, and proteases. Accordingly, in vitro, the secretome of senescent cells increases cell proliferation (23, 24), angiogenesis, and invasion (21). In vivo, it favors the growth of some xenografts (25, 26). An alternative explanation for the existence of the senescence secretome is compatible with the consideration of senescence as a pure intrinsic antitumor barrier. Such consideration is based on subcutaneous xenograft experiments carried out in nude mice. In this model, RAS-transformed hepatoma cells induced to senescence by p53 restoration secrete chemotactic cytokines, including Csf1, Mcp1, Cxcl1, and IL-15, which induce an innate immune response by attracting neutrophils, macrophages, and natural killer cells (7, 27). This inflammatory response leads to complete tumor regression in about 2 weeks due to a prompt clearance of senescent cells. In contrast, p95HER2-induced senescent cells last months in nude mice (Fig. 4). The most likely explanation to reconcile these results is that the composition of the senescence secretome induced by p53 restoration in RAS-transformed hepatoma cells is different from...
that of p95HER2-induced senescent cells. While the former includes cytokines that attracts cellular components of the innate immune system, the latter lacks such cytokines. Supporting this conclusion, we have not detected the expression of Csf1, Mcp1, Cxcl1, or IL-15 in the secretome of p95HER2-induced senescent cells (see Supplementary Tables S1–S3).

Using immunocompetent mice, a recent report shows that the adaptive immune system rapidly clears Ras-induced hepatocarcinoma senescent cells from early tumor lesions (8). However, in line with our conclusions with nude mice, it has been shown that senescent cells expressing an oncogenic form of HER2 are long lived in vivo, also in immunocompetent mice (28). Therefore, these reports also support that clearance of senescent cells in vivo depends on the oncogene that induced senescence, presumably because of the differences in senescence secretomes.

The strict control of the senescence secretome by oncogenes that would reconcile the apparently disparate results aforementioned is strongly supported by different evidence presented in this report. On one hand, the inhibition of HER2 signaling impairs the secretory phenotype of p95HER2-induced senescent cells (Fig. 3). On the other hand, induction of senescence by γ-irradiation leads to a secretome different to that of p95HER2-induced senescent cells, and expression of p95HER2 in irradiation-induced senescent cells results in a secretome similar to that of p95HER2-induced senescent cells (Fig. 3). Therefore, our data show that the composition of the senescence secretome, and thus the cell nonautonomous effects of senescent cells, depends on the specific cause that drives senescence.

In summary, we propose that different oncogenes might lead to senescent cells that, despite showing many common features, are very different with respect to their secretory pheno-
type. The secretome elicited by constitutive HER2 signaling in senescent cell exerts a prometastatic effect that could contribute to the progression of some breast cancers.

Disclosure of Potential Conflicts of Interest
J. Baselga is a consultant/advisory board member of Roche Genentech. No potential conflicts of interest were disclosed by the other authors.

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Correction: Constitutive HER2 Signaling Promotes Breast Cancer Metastasis through Cellular Senescence

In this article (Cancer Res 2013;73:450–8), which appeared in the January 1, 2013 issue of Cancer Research (1), the funding statement omitted a funder. The corrected Grant Support section is given below. The authors regret this error.

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