An Anti-oncogenic Role for Decorin
DOWN-REGULATION OF ErbB2 LEADS TO GROWTH SUPPRESSION AND CYTODIFFERENTIATION OF MAMMARY CARCINOMA CELLS*

Manoranjan Santra, Inge Eichstetter, and Renato V. Iozzo‡

From the Department of Pathology, Anatomy and Cell Biology, and the Cellular Biology and Signaling Program, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania, 19107

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The leucine-rich proteoglycan decorin interacts with the epidermal growth factor receptor and triggers a signaling pathway that leads to growth suppression. We find that decorin causes a functional inactivation of the oncogenic ErbB2 protein in breast carcinoma cells. Upon de novo expression of decorin, the ErbB2 protein is reduced by ~40%, whereas its degree of tyrosyl phosphorylation is almost completely abrogated. Both co-culture experiments or experiments with recombinant decorin demonstrate an initial induction of ErbB2 tyrosine kinase, followed by a profound and long-lasting down-regulation of its activity. This leads to growth inhibition and cytodifferentiation of mammary tumor cells and a concurrent suppression of their tumorigenic potential in vivo. These decorin-mediated effects appear to involve the activation of ErbB4, which in turn would block the phosphorylation of heterodimers containing either ErbB2 or ErbB3. These results provide an explanation for the heightened decorin levels around invasive carcinomas and suggest that decorin may function as a natural antagonist of neoplastic cells enriched in ErbB2.

Central issues in tumor biology are the understanding of the factors that control tumor cell proliferation and the identification of extracellular matrix cues controlling the signaling transducing repertoire that make cancer cells proliferate and invade the host tissues. Among these factors, proteoglycans occupy a central role because of their ability to bind growth factors and to modulate their biological activities (1–5). Decorin, a prototype member of the small leucine-rich proteoglycan family (6–8), is emerging as a powerful modulator of cell growth by affecting several key elements including matrix assembly, growth factor binding, and receptor tyrosine kinase activity (9–11). Decorin levels are suppressed in most transformed cells (12, 13), but markedly increased in post-confluent and serum-starved cells (12, 14, 15) and in the peritumorous stroma of colon cancer (13). The latter may represent a natural biological response of the host cells to the invading neoplastic cells (16). Animals harboring a targeted disruption of decorin and the tumor suppressor p53 exhibit an accelerated rate of death due to invasive thymic lymphomas, suggesting that lack of decorin is permissive for lymphoma tumorigenesis in a mouse model predisposed to cancer (17). Ectopic expression or addition of recombinant decorin induces profound cytostatic effects in a wide variety of transformed cells with diverse histogenetic backgrounds (18, 19). Decorin activates the epidermal growth factor receptor (EGFR)1 kinase, thereby triggering a signaling cascade that leads to a sustained phosphorylation of mitogen-activated protein kinase (MAPK), mobilization of intracellular calcium, induction of the potent cyclin-dependent kinase inhibitor p21WAF1/CIP1, and growth suppression (18, 20–22). Thus, decorin is a novel biological ligand for the EGFR (23), an interaction that could regulate cell growth at the sites of tissue remodeling and cancer invasion (11).

Currently, there are four known receptors for EGF-like ligands constituting the ErbB family of receptor tyrosine kinases (24, 25). Whereas EGFR binds many ligands, including EGF, transforming growth factor-α, epiregulin, and amphiregulin, both ErbB3 and ErbB4 bind to a family of proteins collectively known as neuregulins. A related group of molecules, termed NRG 2, binds to the same two receptors, whereas NRG 3 bind only to ErbB4. Betacellulin and heparin EGF-like growth factor binds to ErbB1 and ErbB4 (26). ErbB2, the most oncogenic member of the ErbB family, binds none of the EGF-like ligands with high affinity (24). However, ErbB2 functions as a shared receptor that binds bivalent EGF-like ligands with low affinity, once they are presented by either one of the high affinity receptors (27, 28). Expression of ErbB2 in the mammary epithelium of transgenic mice induces metastatic disease (29), and amplification/overexpression of ErbB2 is found in approximately 25% of breast cancers and correlates with poor prognosis (30–32). Notably, overexpression of ErbB2 is sufficient to stimulate breast carcinoma migration and invasion of extracellular matrix (33). Owing to its accessible location, ErbB2 is now under intensive scrutiny as a therapeutic target for several types of human tumors (24).

The fact that decorin could affect so many different cell types in which the EGFR could conceivably not be expressed (18) suggested to us that decorin could interact with other members of the ErbB family of receptor tyrosine kinase. We find that de novo expression of decorin leads to a profound inhibition of the steady state levels of ErbB2 phosphorylation in ErbB2-overexpressing breast carcinoma cells. Quantitatively, the overall amount of ErbB2 declines by ~40% in the three independent clones studied in detail; however, the ErbB2 tyrosyl phosphorylation is nearly completely abolished, as well as that of ErbB3 and ErbB4.

1 The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; p21, the cyclin-dependent kinase inhibitor p21WAF1/CIP1; MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; FACS, fluorescence-activated cell sorting; DDR, discoidin domain receptor.

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‡ To whom correspondence should be addressed: Dept. of Pathology, Anatomy and Cell Biology, Rm. 249, JAH, Thomas Jefferson University, 1020 Locust St., Philadelphia, PA 19107. Tel.: 215-503-2208; Fax: 215-923-7969; E-mail: iozzo@iac.cji.tju.edu.
become growth-retarded, with a significant increase in the proportion of cells in the G1 phase of the cell cycle, up-regulate their endogenous levels of p21, undergo in vitro cytodifferentiation, and lose anchorage-independent growth. In addition, the decorin-expressing cells fail to generate orthotopic tumors in nude mice. Co-culture experiments or experiments with recombinant decorin demonstrate an initial induction of ErbB2 tyrosine kinase, followed by a profound and long-lasting down-regulation of its activity. Decorin causes a functional inactivation of the oncogenic ErbB2 by interacting with ErbB4, which in turn would prevent the phosphorylation of heterodimers containing either ErbB2 or ErbB3. These results provide a mechanism by which a naturally occurring protein normally synthesized by fibroblasts and smooth muscle cells, the two key components of the tumor stroma, may counteract the growth of neoplastic cells enriched in oncogenic ErbB2.

EXPERIMENTAL PROCEDURES

Materials and Cells—MDA-453 cells were obtained from American Type Culture Collection. Recombinant human EGF and the EGF domain of herceptin-o were purchased from R&D Systems, Minneapolis, MN. 

Cell Proliferation, Differentiation Assays, Immunohistochemistry, and Orthotopic Tumors—The cells were grown in 48 wells in the concentration of 10⁵ cells/ml. The cells were incubated with the reagent MTS/PMS CellTiter 96™ aqueous non-radioactive cell proliferation assay (Promega) for 30 min in 37 °C, and color reaction was measured according to the company’s protocol. For differentiation assays, tumor cells were plated in multichamber slides (Lab-Tek) and cultured for 4–5 days. The cells were fixed in 10% buffered formalin and stained with Oil Red O to visualize neutral lipids (26). Parallel cultures in multichamber slides were subjected to immunoenzymatic detection of ErbB2 using the monoclonal antibody described above. Bound primary antibody (1:250 dilution) was reacted with unlabeled rabbit anti-mouse bridging antibodies and subsequently with immunocomplexes of alkaline phosphatase and anti-phosphatase monoclonal antibodies. Sections were briefly counterstained with hematoxylin and developed with new fuchsin as the substrate for alkaline phosphatase (37). Two independent clones of MDA-453, clones 4 and 5, were used for in vivo orthotopic tumor growth. Thirty-six female nude (nu/nu) mice (Harlan) were injected with ~10⁶ cells in the lower left mammary region either alone or in combination with Matrigel, an extracellular matrix extracted from Englebreth-Holm-Swarm tumor (38). Tumor development was followed at weekly intervals, and full necropsies were performed on the animals in which tumors developed (17). Animals were maintained in accordance to guidelines of the Institution Animal Care and Use Committee.

Co-culture, Cell Cycle, and Binding Assays—Clone 5, a G418-resistant but decorin-nonexpressing clone, and wild type MDA-453 were cultured in 12-well plates at an initial seeding density of ~10⁵ cells/dish. Decorin-expressing clones 4 and 13 were cultured in microporous inserts (3 μm) and placed on the top of the MDA-453 and clone 5 cells so that the inserts remained within the medium of the target cells. After 4–6 days of co-culture without changes of the medium, the inserts were removed, and proliferation assays were performed according to the manufacturer’s protocol. 

Results

De Novo Expression of Decorin Causes Growth Suppression of MDA-453 Mammary Carcinoma Cells—To test whether other members of the ErbB gene family could be involved in mediating the effects of decorin, we investigated the human MDA-453 mammary carcinoma cells. These cells lack EGFR (41) but overexpress ErbB2 (42), and express low levels of ErbB3 (43) and intermediate levels of ErbB4 (44). Stably transfected clones, which were resistant to G418 (400 μg/ml) for at least 6 weeks of continuous culture, were screened by Northern blotting using decorin cDNA. We detected three clones, which expressed relatively high decorin mRNA levels (Fig. 1A). In all the decorin-expressing clones, the endogenous levels of p21 were concurrently induced. Clone 5, a clone resistant to G418 but lacking decorin expression, and three decorin-expressing clones (clones 4, 13, and 20) were investigated in detail. Decorin proteoglycan was synthesized and released into the conditioned medium by clones 4, 13, or 20 at roughly equal amounts as visualized by Western immunoblotting with specific anti-decorin antibodies (Fig. 1B, left panel). Based on comparison with known amounts of recombinant decorin (35), we calculated that each decorin-expressing clone synthesizes 1.5 μg of decorin/day/10⁶ cells.

Notably, all the decorin-expressing clones were markedly growth-inhibited (Fig. 1C), with the greatest inhibition occurring in clone 4, which expressed the highest levels of decorin, as also demonstrated by anti-decorin immunoblotting of media conditioned by the cells (data not shown). Growth inhibition was time-dependent, and, by day 8, clones 4 and 13 were inhibited by ~75% and 60%, respectively (Fig. 1C). The growth of clone 20 was also inhibited by ~65% (data not shown). The
progressive growth inhibition is likely due to the compound ing effects of decorin, which accumulates in the medium (6). In an independent experiment, the growth of the wild type and clone 5 cells was markedly suppressed by recombinant decorin or decorin core protein (data not shown), in a manner similar to that shown before for colon (20) and squamous (18) carcinoma cells. Finally, FACS analysis revealed a proportional increase in the fraction of the cells in G₁ phase of the cell cycle with a concurrent decrease of cells in G₂ and S phase (Fig. 1C). These experiments were repeated three times with comparable results. Thus, de novo expression of decorin causes growth suppression in these breast carcinoma cells overexpressing the oncogene for ErbB2.

**De Novo Expression of Decorin Causes Phenotypic Differentiation and Prevents Anchorage-independent Growth**—When the wild type and various clones were investigated for their growth in vitro, interesting morphological changes were observed. Whereas the wild type and clone 5 cells were moderately adherent, all the decorin-expressing clones grew as cohesive monolayers or solid sheets, and displayed a flattened morphology with increased cytoplasm (Fig. 2A). The decorin-expressing clones did not form colonies in soft agar as compared with the wild type or clone 5, which formed aggregates >100 cells after 3 weeks in soft agar (Fig. 2B). In addition, the decorin-expressing clones showed intense intracytoplasmic deposits of neutral lipids in contrast to the wild type or clone 5 cells (Fig. 2C). These morphological and phenotypic changes are reminiscent of those observed when the same cells are exposed to epiregulin, a potent pan-ErbB ligand that preferentially activates heterodimeric receptor complexes (26). Notably, epiregulin can activate not only EGFR, but ErbB4 as well, and ErbB3 expression enhances the sensitivity of ErbB4 for activation by epiregulin (45). These results, thus, suggest that decorin may interact with members of the ErbB family other than the EGFR, and that, in doing so, decorin may activate a signaling pathway similar to that activated by epiregulin.

**Decorin Expression Prevents the Formation of Orthotopic Breast Tumors**—To test whether decorin expression would be sufficient to repress orthotopic tumor formation, nude/nu mice were injected with ~10⁶ cells in the lower left mammary region, either alone or in combination with Matrigel, an extracellular matrix extracted from Engelbreth-Holm-Swarm tumor that is known to facilitate tumor take and survival (38). Tumor development was followed at weekly intervals, and full necropsies were performed on the animals in which tumors developed (17). In the absence of Matrigel, none of the animals (n = 20) developed tumors, consistent with the fact that these MDA-453 cells do not form tumors in SCID mice. However, when Matrigel was co-injected with the tumor cells, ~90% of the animals (n = 8) injected with clone 5 cells developed invasive neoplasms following ~6-week latency period. The tumors invaded subcutaneous soft tissues and extended into the pelvic cavity (data not shown). In contrast, none of the animals co-injected with the decorin-secreting clone 4 cells (n = 8) generated any tumors, even 4 months following the end point of the experiments. Thus, decorin expression prevents orthotopic breast cancer formation.

**Decorin Suppresses ErbB2 Activity**—To investigate the mechanism of action of decorin, we cultured wild type and decorin expressing clones to confluence, rendered them quiescent by overnight incubation in serum-free medium and measured the steady state levels of activated (phosphorylated) ErbB2 receptor with specific monoclonal antibody and Western immunoblotting. The results showed undetectable levels of the phosphorylated 185-kDa band corresponding to ErbB2 in all the decorin-secreting clones (Fig. 3A, lanes 3–5, top), while the levels of ErbB2 were only moderately decreased (Fig. 3A, lanes 3–5, bottom panel), as compared with the either wild type (Fig. 3A, lane 2) or clone 5 (data not shown). These experiments were repeated five times, and consistently the steady state levels of ErbB2 in the decorin-expressing clones were reduced to 45 ± 9% of control levels. Only after longer exposures was it possible to visualize low amounts of phosphorylated ErbB2. As a control, we used A431 squamous carcinoma cells that overexpress
EGFR. As expected, a band of ~170 kDa, migrating slightly faster than ErbB2, was observed in A431 cell extracts (Fig. 3A, lane 1, top); this band was confirmed to be EGFR by immunoblotting with monoclonal antibodies against the EGFR (data not shown). Only after long exposure, a small amount of ErbB2 was also detected in the A431 cells (data not shown). Thus, decorin causes a reduction of ErbB2 and an even greater suppression of its tyrosyl phosphorylation.

Because the inhibition of ErbB phosphorylation could conceivably be due to a decorin-mediated activation of a phospha-

**Fig. 3.** *De novo* expression of decorin causes sustained down-regulation of ErbB2 tyrosyl phosphorylation. A, steady state levels of ErbB2 tyrosyl phosphorylation and ErbB2 protein. Confluent cells were serum-starved for 24–28 h prior to lysis. Equal amount of proteins (50 μg/lane) were separated on a 8.5% SDS-PAGE and subjected to Western immunoblotting with monoclonal antibodies against either phosphotyrosine (αPTyr) or ErbB2 (αErbB2). The results shown here are typical of at least five independent experiments in which the level of ErbB2 phosphorylation and ErbB2 protein declined by 95 ± 3% and 45 ± 9%, respectively. B, confluent cells, serum-starved for 24 h, were exposed for 2 h to increasing concentrations of sodium orthovanadate (Na3VO4), a potent inhibitor of phosphotyrosine phosphatase (46), followed by Western immunoblotting with anti-phosphotyrosine monoclonal antibodies. Notice the lack of substantial changes at all concentrations of orthovanadate. In contrast, the phosphorylation of ErbB2 is totally abolished in clones 4, 13, and 20 (the decorin-expressing clones) but is fully maintained in clone 5 or wild type cells (not shown), even at high concentrations (100 μM) of orthovanadate. C, immunohistochemical demonstration of ErbB2 epitopes in wild type and decorin-expressing clones as indicated. Cells were fixed in acetone for 1 min and reacted with the same monoclonal anti-ErbB2 antibody followed by secondary antibody. Original magnification, ×400.
ErbB2 was totally abolished in clones 4, 13, and 20 (the tyrosine phosphatase (46). No significant changes were observed in the constant amount of a nonspecific phosphoprotein of specific monoclonal antibodies as indicated. Equal loading was assured by these cells.

Visualize ErbB2, consistent with the fact that ErbB2 is overexpressed in the decorin-expressing cells. The difference was accentuated by sodium orthovanadate (Na₃VO₄), a potent inhibitor of phosphorylation that involves the EGFR and ErbB2. Thus, we conclude that decorin causes a inhibition of receptor tyrosine phosphorylation of ErbB2, ErbB3, and ErbB4. Confluent cultures (~10⁷ cells) were serum-starved for 24 h prior to lysis. The various ErbB members were immunoprecipitated (IP) from 1 mg of total cell protein, and immunocomplexes were subjected to 8.5% SDS-PAGE and Western blotting (WB) with either phosphotyrosine- or ErbB-specific monoclonal antibodies as indicated. Equal loading was assured by the constant amount of a nonspecific phosphoprotein of ~52 kDa (data not shown). The exposure time required to visualize ErbB3 and ErbB4 was about 8 and 4 times longer, respectively, than that required to visualize ErbB2, consistent with the fact that ErbB2 is overexpressed in these cells.

Because we detected no other phosphorylated band between 160 and 180 kDa (Fig. 3A), corresponding to either ErbB3 and ErbB4, respectively, it is likely that these two ErbB members are also down-regulated by decorin. To formally address this issue, we performed immunoprecipitation studies of quiescent, serum-starved cells with monoclonal antibodies against the three ErbB receptors followed by Western immunoblotting with anti-phosphotyrosine or anti-receptor antibodies. In all cases, there was a total suppression of ErbB2, ErbB3, and ErbB4 phosphorylation (Fig. 4). Interestingly, the levels of ErbB2 were moderately decreased, while the levels of ErbB3 and ErbB4 were essentially unchanged. This may be due to the fact that these breast carcinoma cells overexpress ErbB2 and, thus, are more susceptible to down-regulation as it has been shown in cells overexpressing the EGFR (25;47). Our recent unpublished results have shown that various decorin-expressing clones of A431 cells also show profound suppression of EGFR kinase. Thus, we conclude that decorin causes a inhibition of receptor tyrosine phosphorylation that involves the EGFR and ErbB2.

Collectively, these results indicate that the secreted decorin is capable of inducing the same phenotypic and biochemical changes as the endogenous transgene, thereby eliminating the possibility that the transgene might have affected gene(s) involved in the ErB signaling pathway. The results also demonstrate a role for decorin as a paracrine regulator of tumor cell growth.

Specific Binding of Decorin to the Cell Surface—Next, we sought to determine whether decorin could specifically bind to the cell surface of the mammary carcinoma cells. A radioligand-binding assay was used to analyze the specificity of binding of 125I-decorin protein core to the MDA-453 cells. The binding of the decorin protein core was saturable in the range of 60–80 pmol (Fig. 6A) and specific, since it was nearly abolished by 100-fold molar excess of unlabeled decorin (Fig. 6B). Single-site linear regression analysis, using the method of Scatchard, yielded a $K_d$ of 71 ± 7 nM (Fig. 6C), a dissociation constant remarkably similar to that observed in cell-free binding experiments between decorin and purified EGFR (23). The number of decorin receptor sites was calculated to be 210,000 ± 14,400/ cell. We then utilized similar assays to investigate the decorin binding properties in other cell lines that have different complements of ErbB receptors (Fig. 6D). The largest amount of bound decorin (76 ± 3.5 fmol/mg of cell protein) was observed in A431 squamous carcinoma cells, in line with our previous work, which has shown that decorin binds the EGFR in these EGFR-overexpressing tumor cells (23). A lower amount of bound decorin (31 ± 3 fmol/mg of cell protein) was observed in the breast carcinoma MDA-468, which lacks ErbB2 but expresses EGFR, ErbB3, and ErbB4 (48). The HTC116 colon carcinoma cells, which express lower levels of EGFR (results not shown), also had lower decorin binding activity (35 ± 5 fmol/mg of cell protein).

Thus, we conclude that MDA-453 breast carcinoma cells possess a significant number of decorin-binding sites with affinity constants comparable to those obtained for EGFR-expressing tumor cells.

Decorin Interacts with ErbB4—Because decorin can activate cells that express EGFR as well as the other three members of through the membrane and interact with the target cells (clone 5) cultured in the bottom wells. There was a progressive growth inhibition only in the cells that were co-cultured in the presence of either clone 4 or clone 13 (Fig. 5A). The inhibition of growth was time-dependent and mirrored that observed in the permanently transfected cells. Moreover, after 5 days of exposure to the decorin secreted by clone 4, 13, or 20, the target clone 5 expressed morphological changes and evidence of cytodenervation identical to those described in the decorin-expressing (stably transfected) clones illustrated in Fig. 2 (data not shown). Concurrent with these changes, there was a complete suppression of ErbB2 phosphorylation (Fig. 5B), while the levels of ErbB2 were reduced only by ~40% in three independent experiments. To further confirm that indeed decorin can modulate the ErbB2 signaling pathway, we exposed wild type MDA-453 cells to recombinant decorin (1 μM) for various periods of time. There was a relatively slow, but progressive increase in ErbB2 phosphorylation (Fig. 5C). Quantization of ErbB2 degree of tyrosyl phosphorylation showed a peak at ~2 h followed by a progressive decline (Fig. 5D). By 6 h, ErbB2 phosphotyrosyl levels had reached control values, and by 8 h had declined to ~5% of control levels (Fig. 5D). The suppression of ErbB2 phosphorylation was monitored for an additional 24 h, and was found to be completely suppressed by exogenous decorin (data not shown). Thus, following an initial induction of ErbB2 phosphorylation, decorin causes a pronounced and long-lasting down-regulation of ErbB2 phosphorylation.

FIG. 4. Ectopic expression of decorin causes a sustained block of tyrosyl phosphorylation of ErbB2, ErbB3, and ErbB4. Confluent cultures (~10⁷ cells) were serum-starved for 24 h prior to lysis. The various ErbB members were immunoprecipitated (IP) from 1 mg of total cell protein, and immunocomplexes were subjected to 8.5% SDS-PAGE and Western blotting (WB) with either phosphotyrosine- or ErbB-specific monoclonal antibodies as indicated. Equal loading was assured by the constant amount of a nonspecific phosphoprotein of ~52 kDa (data not shown). The exposure time required to visualize ErbB3 and ErbB4 was about 8 and 4 times longer, respectively, than that required to visualize ErbB2, consistent with the fact that ErbB2 is overexpressed in these cells.
the ErbB family of receptor tyrosine kinase, it was necessary to establish which receptor would specifically mediate the signaling driven by decorin. To this end, we utilized a series of NIH 3T3 cells stably transfected with individual ErbB receptors (49). Unlike their parental wild type cells, these NIH 3T3 7d variant clones do not express detectable levels of any known ErbB subfamily members of receptor tyrosine kinase (49). Thus, they represent a good model to study specific interactions with diverse ligands including heparin binding-EGF (50) and heregulin (51). Moreover, these cells have been successfully used to investigate the formation of EGFR/ErbB4 heterodimers (51).

Cells were rendered quiescent by serum starvation for 24 h and then challenged with a 10-min incubation in the absence or presence of EGF (100 ng/ml). The latter binds to ErbB3 and ErbB4 and transmodulates EGFR and ErbB2 via the binding receptor (44). Concurrently, cells were incubated for 40 min with recombinant decorin (1 mM). When cells were incubated with decorin, it became apparent that decorin activated only EGFR and ErbB4 (Fig. 7). Notably, in the ErbB2 transfectants, the ErbB2 was constitutively tyrosine-phosphorylated and unresponsive to either EGF or decorin. These results are identical to those obtained with an independent clone of NIH 3T3 7d cells stably expressing ErbB2, which were unresponsive to heregulin-β2 (51). In agreement with previous results (49), the phosphorylation of ErbB3 was very weak, since it required 5 times longer exposure to be detected. This is consistent with the fact that several amino acids, usually conserved in the ErbB family members, are altered in ErbB3 (29), and that ErbB3 exhibits impaired kinase activity as compared with the EGFR (52). Thus, the decorin-induced down-regulation of ErbB2 receptors is likely mediated by heterodimerization of ErbB4 with ErbB2.

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FIG. 5. Decorin causes a transient activation followed by a prolonged down-regulation of ErbB2 kinase. A, growth of co-cultures in which the decorin-secreting clones (clones 4 and 13) were cultured in the top chamber of a microporous 3-μm diameter well, while the nonexpressing clone 5 was cultured in the bottom well. Note the progressive growth inhibition only in the cells that are co-cultured in the presence of either clone 4 or clone 13. B, Western immunoblotting of total cell lysates (~50 μg of total cell protein/lane) using monoclonal antibodies directed against either phosphotyrosine (αPTyr) or ErbB2 (αErbB2). The various combinations are noted in the top. C, confluent cultures of MDA-453 cells were serum-starved for 24 h and incubated for the designated times with or without recombinant decorin (1 μM). D, quantization by scanning densitometry of the degree of tyrosyl phosphorylation of the ErbB2 in two independent experiments. Each value represents the mean ± S.D. of triplicate determinations.

FIG. 6. Decorin binds to the cell surface of MDA-453 mammary carcinoma cells. A, saturation curve for the binding of 125I-labeled decorin core protein to the surface of MDA-453 mammary carcinoma cells. The values represent the average of two independent experiments run in triplicate. B, specificity of the 125I-labeled decorin core protein binding as determined by incubating the cells with cold decorin as indicated. The values represent the average of two independent experiments run in triplicate. The nonspecific binding (i.e. absence of cells) was 1020 ± 260 cpm (n = 15). C, Scatchard plot for the binding of 125I-labeled decorin core to MDA-453 cells. The cumulative data from five independent experiments gave a Kd = 71 ± 7 nM. D, quantitative binding of 125I-labeled decorin core to various cells using 125I-labeled decorin core. The values represent the mean of two independent experiments run in triplicate.

The ErbB family of receptor tyrosine kinase, it was necessary to establish which receptor would specifically mediate the signaling driven by decorin. To this end, we utilized a series of NIH 3T3 cells stably transfected with individual ErbB receptors (49). Unlike their parental wild type cells, these NIH 3T3 7d variant clones do not express detectable levels of any known ErbB subfamily members of receptor tyrosine kinase (49). Thus, they represent a good model to study specific interactions with diverse ligands including heparin binding-EGF (50) and heregulin (51). Moreover, these cells have been successfully used to investigate the formation of EGFR/ErbB4 heterodimers (51).

DISCUSSION

This study extends our previous work showing that decorin is a powerful inhibitor of growth in a wide variety of tumor cells with diverse histogenetic backgrounds (18, 20) and that this effect is mediated by a specific interaction of decorin core with the EGFR (21–23). We have now discovered that decorin leads to a protracted down-regulation of another member of the ErbB
family of receptor tyrosine kinases, namely ErbB2. In response to secreted decorin, the mammary carcinoma cells become growth-impaired and show signs of cytodifferentiation culminating in the block of tumorigenicity both in vitro and in vivo.

Decorin Is an Anti-oncogenic Protein—Specialized signal transduction systems allow cells to sense the microenvironment and to respond to stimuli by adjusting gene expression. Proteoglycans play fundamental roles in cancer biology, not only as modulators of growth factor activity (4, 53), but also as a physical and bioactive barrier to the invading neoplastic cells (11). This study expands this concept by providing evidence that a leucine-rich proteoglycan can act as a pan-ErbB ligand and, in doing so, down-regulate the activity of one of the most potent oncogenic proteins, ErbB2, whose overexpression is linked to poor prognosis and increased cancer mortality in breast, ovary, and prostate (30, 31, 54). Ligand-receptor complexes that contain ErbB2 are more potent than other receptor combinations because ErbB2 causes conversion from low to high affinity growth factor binding sites (54). Moreover, the internalization of ErbB2 is lower than EGF-R. Thus, the higher affinity binding sites, together with the decreased internalization rate, would result in a receptor-ligand complex that resides longer at the plasma membrane and prolong the duration of receptor signaling (25, 54). Although modulation of ErbB2 kinase activity and substrate specificity by antibodies against its extracellular domain has been known for some time, this is the first demonstration of a proteoglycan core protein affecting the biology of ErbB2. Decorin is a powerful stimulus that triggers a signaling cascade that leads to growth arrest in a multitude of transformed cells (18). Our data support a model by which decorin would interact with ErbB4, which, in turn, would form heterodimers with ErbB2, thereby suppressing ErbB2 kinase activity. This would lead to a protracted induction of p21 and eventually to growth inhibition.

We provide several lines of evidence that decorin is directly involved in ErbB2-mediated signaling events in mammary carcinoma cells. First, ectopic expression of decorin causes growth inhibition, induces endogenous p21, and causes arrest of the cells in G1. Second, the decorin-expressing clones do not grow in suspension, develop into cohesive monolayers, appear contact-inhibited, and display a morphology consistent with cytodifferentiation toward a secretory phenotype. Third, the decorin-expressing cells do not form orthotopic tumors in nude mice, and exposure of the wild type cells either to the secretions of the decorin-expressing cells or to recombinant decorin causes the same biochemical and phenotypic changes induced by the transgene. Fourth, there are numerous and specific binding sites for decorin in the breast carcinoma and other tumorigenic cells, indirectly establishing the concept of a specific receptor for decorin. Whether decorin interacts with another, not yet identified, surface protein that in turn affects ErbB biology needs to be addressed in future studies. The action of decorin is evocative of hereceptin, a humanized monoclonal antibody directed toward the extracellular domain of ErbB2 and capable of blocking the ErbB2 kinase activity in cells overexpressing the oncogene (54). Similar to decorin-mediated induction of p21, hereceptin causes an augmentation of a cyclin-dependent kinase inhibitor p27Kip1 and the retinoblastoma-related protein p130, both of which prevent the cells from traversing the S phase (54).

Extracellular Matrix Proteins Interact with Receptor Tyrosine Kinases—Following the discovery that discoidin domain receptors (DDR) 1 and 2, two orphan receptor tyrosine kinases, are the receptors for fibrillar collagen, views on how matrix molecules affect cell behavior have been reconsidered (55, 56). Stimulation of the DDR tyrosine kinase differs from traditional growth factor/receptor signaling since the kinetics of activation are much slower and protracted in time, similar to the decorin/ErbB interplay reported here. In reaction to collagen-mediated activation of DDR2, there is enhanced collagenase (MMP-1) expression, which leads to a negative feedback loop that controls extracellular levels of collagen (55). Because decorin is intimately associated with fibrillar collagen, a complex scenario where multimeric interactions might take place should be entertained. Specificity can occur at the cellular level, since ErbB2 and DDR expression are quite different. Moreover, the kinetics of decorin-induced ErbB2 activation differ from those induced by EGF or other ligands, which exhibit fast binding and internalization followed by rapid down-regulation of the receptor (47, 57).

Decorin binds to ErB receptors in a fundamentally different manner than traditional growth factors. First, the decorin binding is a much lower affinity, $K_d = 70–90 \text{ nM}$ versus $0.1–1 \text{ nM}$ for EGF. Second, it appears that the binding is sustained since MAPK/extracellular signal-regulated kinase activation and p21 induction is protracted (21, 23). This may indicate that receptor activation is mediated by a rate-limiting step that is determined by a weak interaction between decorin and its cognate receptor(s) and a slow on-rate for binding of decorin to its receptor(s). Notably, a model has been proposed that shows cells can enact a differentiation program, as in the decorin case, or a proliferative response to receptor tyrosine kinases solely on the basis of the duration of MAPK activation (58). Accordingly, sustained activation of MAPK leads to different cellular responses than transient activation, since the former causes nuclear accumulation of the active MAPKs and subsequent phosphorylation of transcription factors. Implicitly, cellular responses will depend on the types of transcription factors expressed by a given cell, thus providing a layer of specific control. Quantitative differences in MAPK activation can, therefore, be translated into qualitative changes (58).

A major conclusion of our findings is that the composition of the extracellular matrix has profound effects on the growth of infiltrating tumor cells by directly affecting the transcription of regulatory molecules via the ErbB family members. This concept is based on the observation that extracellular matrixes are relatively stable and long-lived. For example, the turnover rate of collagen and other proteoglycans can be weeks or months. Unlike brief (minutes to hours) treatments of most published experimental systems, prolonged stimulation (days to weeks) with a single ligand can lead to a re-
marked shift in activity of a receptor. Such shifts are likely to play key roles in the smooth regulatory milieu of complex tissues in adult organisms.

Potential Mechanisms of Action of Decorin—Our results show that specific interactions between decorin protein core and cell surface receptor tyrosine kinases of the ErbB family leads to down-regulation of constitutively activated ErbB2 and EGFR (results not shown). We believe that these responses are physiologically relevant and can occur in vivo because the concentration of decorin used in our studies (1–2 μM) is lower than that estimated to occur in collagenous matrices (50–125 μM), the site of the interaction between mammary tumor cells and the tumor stroma.

Besides the recruitment of tyrosine phosphatases or the negative c-Cbl, endocytosis of ligand-receptor complexes is a main process for the incremental decline of growth factor signaling (26, 59, 60). In general, endocytosis of the ErbBs requires activation of their kinases and autophosphorylation (47), both of which are induced by decorin (21, 23). Decorin could deact-
ivate ErbB2 signaling by various mechanisms. First, it might bring to the vicinity of the ErbB2 a transmembrane tyrosine (26, 59, 60). In general, endocytosis of the ErbBs requires and the tumor stroma.

concentration of decorin used in our studies (1–2 m)
M), the site of the interaction between mammary tumor cells and the tumor stroma.

In conclusion, the results of our study have direct implications in the biology of breast tumor stroma as a natural barrier to cancer spread. Decorin is a naturally occurring proteoglycan that accumulates at the sites of tumor invasion, is induced by the same drugs (i.e., corticosteroids) used to combat tumor growth (67), and induces p21, a potent inhibitor of cyclin B-dependent kinases. The previous and current data provide a mechanistic framework for understanding how this prototype member of leucine-rich proteins affects the biology of ErbB receptors. The results in Drosophila have established that the EGFR pathway is disrupted by kekkon1, a molecule homolo-

gous to decorin, further stressing the importance of this con-
served biological pathway in ErbB signaling.

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Decorin Blocks ErbB2 Activity