Inhibition of HER-2(neu/ErbB2) restores normal function and structure to polycystic kidney disease (PKD) epithelia

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Abstract

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a very common lethal monogenetic disease with significant morbidities and a high likelihood of progression to renal failure for which there is no proven disease-specific therapy currently available for clinical use. Human ADPKD cystic epithelia have proliferative abnormalities mediated by EGFR over-expression and mispolarization leading autocrine response to EGF family ligands. We now show that apical localization of EGFR complexes in normal fetal and ADPKD epithelia is associated with heterodimerization of EGFR(HER-1) with HER-2(neu/ErbB2), while basal membrane localization in normal adult renal epithelia is associated with EGFR(HER-1) homodimers. Since ADPKD epithelial cells have reduced migratory function, this was used as a bioassay to evaluate the ability of compounds to rescue the aberrant human ADPKD phenotype. General tyrosine kinase inhibition by herbimycin and specific inhibition of HER-2(neu/ErbB2) by AG825 or pretreatment with ErbB2 siRNA reversed the migration defect of ADPKD epithelia. Selective inhibition of EGFR(HER-1) showed partial rescue. Increased ADPKD cell migration after inhibition of p38MAP kinase but not of PI3-kinase implicated p38MAPK downstream of HER-2(neu/ErbB2) stimulation. Daily administration of AG825 to PKD1 null heterozygous mice significantly inhibited the development of renal cysts. These studies implicate HER2(neu/ErbB2) as an effector of apical EGFR complex mispolarization and that its inhibition should be considered a candidate for clinical therapy of ADPKD.

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1. Introduction

Polycystic Kidney Disease (PKD) is a group of diseases caused by mutations in single genes [1]. The most common and clinically significant of these is ADPKD, a progressive cystic disease leading to endstage renal failure that is estimated to affect 10 million patients world-wide. Although there has been a significant increase in our understanding of the basic biology of ADPKD in recent years, this has not yet been translated into an effective therapy to delay the onset or reduce the rate of decline of renal function in these patients. Although renal cysts may develop in utero [2], it is their subsequent expansion to occupy >66% of the grossly enlarged kidney that leads to the onset of renal functional decline, hypertension and other associated symptoms which do not usually occur until adulthood and typically in the 4th or 5th decade. The progressive nature of this disease and the ability of the kidney to function adequately with >30% normal renal parenchyma presents a substantial window of opportunity to treat by retardation of cystic expansion.

The cell biology of cyst expansion in ADPKD is understood to involve a combination of hyper-proliferation and fluid secretion in every segment of the nephron that is brought about in part, by defects in the polarized distribution and function of epithelial membrane proteins including the epidermal growth factor receptor (EGFR) and the sodium pump, as well as cAMP-dependent chloride transport [3–5]. Apical mislocalization, over-expression and hyperactivity of the EGFR protein complex, coupled with apical secretion of EGF and related ligands into cyst lumens, has been shown to create an autocrine/paracrine proliferative loop in human ADPKD epithelia in vivo...
and in vitro as well as in mouse models of ADPKD and autosomal recessive (AR)PKD [3,4,6] (Fig. 1). Importantly, EGFR receptor blockade, restriction of bioligand availability or genetic crosses with waved mice (deficient in EGFR) have been shown to have beneficial effects in mice with ARPKD [7–9]. In addition mice transgenic for activated EGFR or for Her-2(neu-ErbB2) develop renal cystic disease [10]. EGFR (HER-1) belongs to a larger family of molecules of which there are 4 known members: HER (neu/ErbB) 1, 2, 3 and 4 that are capable of homo- and hetero-dimerization, regulation and modulation by several ligands and cross-talk between heterologous signaling pathways [11,12]. In addition to essential roles as mediators of cell proliferation and differentiation in development, increased levels and activation of EGFR(HER-1) or HER-2(neu/ErbB2) have been reported in several human malignancies including those of the breast, pancreas, lung, head and neck, colon, prostate, stomach and ovaries, and the kidney and predicts an aggressive course of disease and poor prognosis [13,14].

Since we had previously demonstrated that ADPKD is often associated with the failure to down-regulate fetal genes including Pax-2, WT-1 and the β2 subunit of NaK-ATPase [15,16], that the mispolarization defects of NaK-ATPase are due to the persistent expression of the fetal form (α1β2) of NaK-ATPase [17,18]; and that abnormal molecular species of EGFR-like proteins are present in ADPKD kidney tissues [3], the present studies sought to determine whether the aberrant apical localization of the EGFR complex seen in ADPKD cystic epithelia was due to the persistent expression and heterodimerization of EGFR (HER-1) with a fetal isoform. This was confirmed by the cloning and localization of ErbB2 in ADPKD and fetal renal epithelial cells as well as by co-immunoprecipitation analyses. The importance of ErbB2 as a potential therapeutic target for ADPKD was further suggested since its inhibition by small molecule or siRNA treatment restored normal migratory function to human ADPKD epithelia in an in vitro assay and retarded renal cyst formation in PKD null mice.

2. Methods

2.1. Tissues and cells

Normal kidneys were procured by National Disease Research Interchange (Philadelphia, PA). Only kidneys without disease that had not been subjected to warm ischemia prior to being flushed with Collins salts solution at 4 °C were selected. These kidneys had been prepared for transplantation but rejected for

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Fig. 1. Schematic of EGF Receptor-mediated cystic expansion in ADPKD kidneys. (A) Normal renal tubule epithelia have EGF Receptor complexes on basal cell membranes. (B) ADPKD cystic epithelia have apical EGF Receptor complexes and secrete EGF apically into the lumen. (C) EGFR(HER-1)/HER-2 (neu/ErbB2) heterodimers are localized on apical cell membranes of normal fetal and ADPKD cystic epithelia. EGFR(HER-1) homodimers are localized on basal membranes of normal adult renal tubule epithelia.
surgical or matching reasons. ADPKD kidneys were procured at the time of surgical removal prior to transplantation.

Primary cell strains were derived by microdissection of normal human adult kidney collecting tubules and cyst-lining epithelia from ADPKD kidneys attachment and growth on type I collagen in 1% serum-containing fully defined supplemented media [19,20]. Conditionally immortalized (Cl) normal and ADPKD cell lines were derived from primary cultures of individually microdissected collecting tubules and ADPKD cyst epithelia by retroviral transduction of neomycin-resistance and temperature sensitive T antigen, into monolayers during the exponential phase of growth followed by selection for 6 weeks in Geneticin [21] and dilution cloning. Functional and marker analysis have previously shown that these cultured lines attain maximal differentiated properties after 5–13 days at 37 °C [20]. Clones that recapitulated the EGFR (HER-1) and HER-2 (neu/ErbB2) protein expression and distribution patterns seen in vivo most closely were selected for use in these studies.

Wild-type (+/+) and heterozygous (+/−) PDK1 null mice [22] were identified by genomic PCR and 4 groups of 5 animals each were allowed to develop normally for up to 4 months. Test agents were added to the drinking water of the mice from 6 weeks of age. None had an effect on the amounts of water drunk by the mice. One group of +/− mice received the highly selective ErbB2 inhibitor, AG825 (50 µM, Calbiochem); one group of +/− mice received DMSO vehicle (1:1000); while one group of +/+ and one group of +/− mice had no additives in their drinking water. MRI analysis was carried out 4 weeks prior to sacrifice when kidneys were removed and quantitative histo-pathological analysis of 4% paraformaldehyde fixed kidneys stained with hematoxylin and cosin was carried out. Whole kidneys (n=10 in each group) were embedded, and 10×1 mm, evenly spaced slices were obtained by vibrotome sectioning throughout the kidney. After re-embedding, 10×5 µm sections were obtained from each slice (n=100 sections per kidney) for quantitative analysis of tubule diameters. Digital images of non-overlapping (571×428 µm) fields were taken for each section. A counting grid with 8000 squares was overlaid and every eighth square was analyzed for tubule diameter using Image J—Grid Plugin (rsb.info.nih.gov/ij).

2.2. RT-PCR degenerate cloning of Her2(neu/ErbB2)

1024x degenerate 17-mer sense and 512x degenerate 17-mer antisense primers were designed against conserved regions of the tyrosine kinase domain of EGFR family proteins aligned using the UWGCG Pileup program and were used to screen a human fetal kidney library. Multiple cDNA clones of the EGF receptor homolog HER-2(neu/ErbB2) were obtained from human fetal and ADPKD kidney.

2.3. Immunohistochemistry

Tissues were sliced and fixed at source for 4 h at 4 °C in 4% paraformaldehyde in Phosphaflered Saline (PBS), dehydrated and embedded in low temperature paraffin prior to sectioning at 5 µm. Monolayer cell cultures were fixed in 4% paraformaldehyde in PBS for 10 min. Immunolocalization was carried out using an indirect avidin–biotin-enhanced technique (Vectorstain, Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazole detection yielding a red reaction product (Vector). For each experiment, control sections were incubated in Vector Laboratories) and aminoethylcarbazol...
By contrast, in ADPKD as well as normal fetal kidneys, both EGFR and HER-2/neu were localized at the apical plasma-membranes of cyst lining epithelia and fetal ureteric bud-derived collecting tubules, respectively (Fig. 2A, C, D, F). This suggested that the persistent expression of renal HER-2(neu/ErbB2) after birth was associated with the apical distribution of the EGFR complex in ADPKD epithelia that leads to receptor complex accessibility to apical EGF ligand and increased proliferation.

3.3. EGFR(HER1) and HER2(neu/ErbB2) heterodimerize in ADPKD and fetal epithelia

To prevent contamination from non-epithelial cell types, characterized differentiated clonal human renal epithelial cell lines were used that recapitulated the patterns of EGFR and HER-2(neu/ErbB2) expression seen in vivo. Western immunoblot analysis detected EGFR (HER-1) in normal fetal, normal adult and ADPKD cells and HER-2(neu/ErbB2) in normal fetal and ADPKD cells only (Fig. 3 right panel). Immunocytochemistry confirmed apical distribution of EGFR and HER-2(neu/ErbB2) in ADPKD and normal fetal epithelia but basolateral staining for EGFR only in normal adult epithelia (Fig. 3A–F). To determine the subunit composition of EGFR complexes in these epithelia, Co-immunoprecipitation analysis showed that EGFR(HER-1) co-associated with HER-2(neu/ErbB2) in ADPKD and human fetal (HFCT) epithelia but no heterodimers were detected in normal human adult tubule epithelia (NHCT) (Fig. 4).

3.4. Inhibition of HER-2(neu/ErbB2) rescues the abnormal migratory defect of human ADPKD epithelia in vitro

ADPKD epithelia have been shown to exhibit many alterations by comparison to age-matched normal renal tubular epithelia, e.g., NHCT, including significantly impaired migration in response to growth factor gradients by comparison to age-matched normal human renal epithelia (Fig. 5A). Since this property is consistent between primary (1°) cultures from different individuals and derived conditionally immortalized (CI) cell lines, this phenotypic property provides a unique and rapid screening assay to evaluate the effects of potential modulators of the human ADPKD phenotype. Since previous studies had implicated hyperactivity of the EGFR tyrosine kinase as a mechanism for cyst enlargement [3,7], the effect of the general tyrosine kinase inhibitor, Herbimycin was first evaluated and shown to return the ADPKD migration curve to normal (Fig. 5B). Protein tyrosine phosphatase inhibition by okadaic acid or sodium vanadate, however, either had no effect or further decreased ADPKD epithelial cell migration (Fig. 5B). To determine the relative efficacies of EGFR antagonists, a variety of compounds were compared. Of interest and significance, the addition of a specific, selective inhibitor of HER-2(neu/ErbB2), AG825 was shown to provide the most effective rescue of the ADPKD migration defect (Fig. 5B). The selective EGFR inhibitor AG1478 also provided significant improvement while the tyrophostin EGFR inhibitors A25 and B42, which show additional cross-reactivity against JAK2, ERKs and phospho-FAK were less effective (Fig. 4C). No
alterations in proliferation were observed in these assays, most probably due to the lack of T antigen at 37 °C and the short periods of exposure to the drugs. Further inhibitor analysis suggested that the rescue of the ADPKD phenotype could be mediated via the p38 MAP kinase pathway since the ADPKD migration defect was rescued by its inhibitor SB203580 but not after inhibition of MEK 1 and 2 by UO126 nor by inhibition of the PI3 kinase pathway by Wortmannin (Fig. 5D). No additive effect of AG825+SB203580 was observed (data not shown) suggesting that maximal migration had already been reached under the conditions of this assay. Finally, occupancy of the EGFR/HER-2(neu/ErbB2) receptor dimer by EGF or Heregulin ligands depressed ADPKD migration further (Fig. 4E) confirming a primary role for receptor occupancy and activation in ADPKD cell aberrant function.

To further confirm the central role for HER-2(neu/erbB2) as a mediator of ADPKD epithelial malfunction ADPKD epithelial cells were treated with a specific ErbB2 siRNA which reduced ErbB2 protein levels by approximately 70% (Fig. 6A). After 16 and 36 h, the siRNA-treated cells showed significantly increased migration in response to growth factor gradient by comparison to the control cells: 61 ±4.9 versus 45± 3.7, \( P<0.05 \) and 91 ±7.3 versus 65± 5.2, \( P<0.05 \), respectively (Fig. 6B). This further confirms that reduction in ErbB2 expression leads to restoration of normal phenotypic function to human ADPKD epithelial cells.

3.5. HER-2(neu/erbB2) inhibition retards cyst formation in PKD1 null mice

In addition to human cell based assays, it is important to test the effects of potential therapeutic manipulations in relevant mouse models of disease. For this evaluation the PKD1 null mouse was selected, since in its heterozygous state renal collecting tubule cysts are seen after 8 weeks of age (Fig. 7A), closely mimicking human ADPKD. It had previously been reported that EGFR is apically mislocalized in the cysts of
PKD1 knockout mice [22] and Western immunoblot analysis confirmed ErbB2 expression in fetal and cystic +/- mice (Fig. 7C), an analogous situation to that seen in human kidneys (compare with Fig. 2). Quantitative analysis of 10 kidneys from 5 mice each in 4 groups of normal (wild-type) and heterozygous PKD1 null mice showed that treatment with AG825 reduced the appearance and degree of cystic expansion of renal collecting tubule cysts as determined by stereological measurement of collecting tubule diameters (Fig. 7B). These measurements reached statistical significance and represents a beneficial effect in the very early stages of cyst formation in these mice. This confirms that inhibition of ErbB2 results in therapeutic benefit.

PKD1 null mice showed that treatment with AG825 reduced the appearance and degree of cystic expansion of renal collecting tubule cysts as determined by stereological measurement of collecting tubule diameters (Fig. 7B). These measurements reached statistical significance and represents a beneficial effect in the very early stages of cyst formation in these mice. This confirms that inhibition of ErbB2 results in therapeutic benefit.

![Image](image-url)
in a mouse model of ADPKD. Additional measurements of body, heart and kidney weight ratios in treated +/− versus untreated +/− and wildtype +/+ control mice did not show any weight loss or adverse effects on behaviour in these mice (data not shown).

4. Discussion

Increased proliferation is an important component of cystic expansion in ADPKD and is understood to be brought about, at least in part, by the apical mislocalization of EGFR complexes noted in cystic epithelia from human and numerous mouse and other models of PKD [3,4,6,22].

The EGFR family of receptors is comprised of 4 members. Each protein is made up of an extracellular ligand binding domain, a single transmembrane domain and an intracellular portion containing the catalytic core and regulatory binding sites. On binding of an appropriate ligand of the EGF family, the membrane receptor molecules dimerize prior to activation by autophosphorylation of specific tyrosine residues in the intracellular domain. This creates binding sites for SH2 and PTB-domain-containing effector and adaptor proteins and leads to activation of the Ras/MAP kinase signaling pathway followed by a proliferative response. Members of the EGFR family frequently form heterodimers. EGFR/HER-2(neu/ErbB2) heterodimerization has been shown to reduce EGFR complex internalization by endocytosis [23] and thus contribute to its hyperactivity. These properties have also been noted in PKD epithelia [24].

Epithelial cell adhesion and migration are fundamental components of the 3-dimensional morphogenesis during normal kidney development and differentiation. Inhibition of PKD1-encoded polycystin-1 function during development results in decreased migration and cystic dilation of collecting tubules [25]. Apparently, these alterations in epithelial cell behaviour are maintained since human and mouse PKD cyst lining epithelia also show characteristically increased adhesion and decreased directional migratory responses.

Our studies show that HER-2 (neu/ErbB2) is highly expressed in renal tubule epithelia of the normally developing kidney as well as in ADPKD cyst lining epithelia. However, no expression of HER-2(neu/Erb-B2) was seen in normal adult kidneys. In addition, although the localization of EGFR is restricted to the basolateral membranes of normal adult renal tubule epithelia, EGFR and HER-2(neu/Erb-B2) were seen on the apical plasma membranes of both normal fetal renal tubules and of ADPKD cyst-lining epithelia. These observations suggested that localization and activities of EGFR (HER-1) and HER-2(neu/Erb-B2) at specific polarized membranes of the tubular epithelial cell surface are essential for normal renal development and maintenance. In addition, these studies suggested that the persistent expression of renal HER-2(neu/Erb-B2) after birth might be associated with the apical distribution of the EGFR complex in ADPKD epithelia that would then lead to EGFR/HER-2(neu/Erb-B2) complex accessibility to apical EGF ligands and result in increased proliferation. Heterodimerization of EGFR(HER-1) with HER-2(neu/Erb-B2) in normal fetal and ADPKD epithelia was confirmed by co-immunoprecipitation analysis.

Based on these observations that both HER-2(neu/ErbB2) expression and heterodimerization with EGFR(HER-1) were abnormal after birth in ADPKD and that its continued expression was associated with the deleterious apical mislocalization of functional EGFR complexes in ADPKD cysts, HER-2(neu/ErbB2) inhibition was evaluated as a mediator of rescue of the ADPKD cystic phenotype. ADPKD epithelia have been shown to exhibit many alterations by comparison to normal renal tubules including proliferation, secretion, adhesion and migration ([1] for review). Using a robust cell-based
migration assay, in which human ADPKD epithelia migrate through membranes orders of magnitude more poorly than normal age-matched renal epithelia, in response to a growth factor gradient, it was shown that specific inhibition of HER-2 (neu/Erb-B2) by small molecule (AG825) or specific siRNA was highly effective in restoration of normal migratory function. Similar results with Herbimycin and SB203580 suggest that this is due to its tyrosine kinase activity mediated via the p38 MAP kinase pathway. Of interest, EGFR(HER-1)-specific inhibitors were also partially effective, but less so when cross-reactive with other intracellular pathways including JAK2, MEK/ERKs or PI3 kinase.

These results therefore implicate HER-2(neu/ErbB2) in addition to EGFR (HER1) as a new target for therapeutic retardation of cystic expansion in human ADPKD since normal epithelial function can be restored to human ADPKD epithelial cells in vitro. Importantly also, cystic development in heterozygous PKD1 mutant mice was significantly reduced by administration of the same HER-2(neu/Erb-B2) inhibitor to their drinking water from 6 to 16 weeks. Since increased levels of EGFR and/or HER-2 (neu/ErbB2) are characteristic of several types of solid tumors including those of the breast, lung, pancreas and kidney, this is already an active area of targeted drug development and shows much promise. Both small molecule inhibitor and antibody approaches have been used to target these receptor signaling pathways including Herceptin (Genentech-Roche), a humanized antibody recognizing the extracellular domain of the HER-2(neu/ErbB2) receptor; Erbitux (Imclone) a humanized antibody that binds the EGF (HER-1); Tarceva (OSI-Genentech-Roche) and Iressa (Astra -Zeneca) which inhibit EGFR tyrosine kinase activity. The potential utility of specific tyrosine kinase inhibitor therapy has recently been emphasized by the successful development of Gleevac (Novartis) which inhibits intracellular signaling by the mutant fusion protein Bcr-Abl non-receptor tyrosine kinase found in chronic myelogenous leukemia [26]. Small molecule therapies have the advantage of oral administration of one tablet per day and of fewer, relatively minor side effects. The availability of these FDA approved, so-called “smart drugs” that specifically target HER-1/HER-2 receptor/ligand interactions, tyrosine kinase activation or downstream signaling pathways and their proven effectiveness in highly proliferative cancers suggests a possibility that the same drugs might be effective at lower daily doses in the HER-1/HER-2-mediated but less proliferative condition of ADPKD. Since Iressa has been shown to be most effective in those patients with a specific EGFR mutation [27], it can also be speculated that different drugs targeting EGFR/HER-2(neu/ErbB2) might be differentially effective in specific patients depending on their specific disease gene (PKD1, PKD2) mutation, or degree of dependence on EGFR or HER-2(neu/ErbB2) activation.

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Appendix A. Supplementary data


References


