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FGF RECEPTORS AS TARGETS FOR DRUG DEVELOPMENT

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BACKGROUND AND RESULTS

Clinical Applications for the FGFs

The fibroblast growth factor (FGF) family is comprised of at least seven closely related small proteins (15-29 kDa) which stimulate growth of a wide variety of cells of mesenchymal, epithelial, and neuroectodermal origin (1). The first FGFs to be characterized in any detail and cloned are acidic FGF (aFGF) (2) and basic FGF (bFGF) (3) which were purified by several different assays including promotion of cell growth and induction of angiogenesis (1). The newest member of the FGF family, keratinocyte growth factor (KGF), was purified and eventually cloned on the basis of its ability to selectively promote the growth of epithelial cells (4).

In contrast, the remaining four FGFs were identified on the basis of their ability to transform normal cells: hst/KFGF was isolated as a transforming gene from human stomach tumor and Kaposi's sarcoma DNA (5,6); the int-2 gene is activated by the nearby insertion of mouse mammary tumor virus (7); FGF-5 was isolated as a transforming gene from bladder carcinoma (8) and FGF-6 was isolated by extensive homology to hst/KFGF (9). It is worth noting that aFGF and bFGF are also able to act as transforming factors when overexpressed in appropriate cell lines (10,11). It is possible that the transforming activity of the FGFs can contribute to human cancer, since the hst/int-2 locus is amplified in human breast and esophageal carcinomas (12-15).

The FGFs are important in normal embryonic development since they induce the early stages of mesoderm formation (16-18) and are expressed in temporal and spatial specific patterns during embryogenesis (1). In addition, the FGFs serve as neurotrophic agents and survival factors for neuronal cells (19,20). All of the FGFs exhibit high binding affinity towards heparin which, in addition to playing an important role in the animal, allows easy purification of the growth factors.

Since the FGFs promote angiogenesis and growth, survival and migration of most cell types, they have been proposed as general wound healing agents and for the specific conditions of corneal ulcers and bone fracture repair. Although these represent therapeutic areas for FGFs as agonists, there are many other areas where FGF antagonists might be particularly useful. These include conditions of inappropriate angiogenesis such as diabetic retinopathy and solid tumor vascularization, and conditions of FGF overexpression in primary human tumors (12-15) and benign prostatic hypertrophy (21). Rheumatoid arthritis, which is a complex pathology, exhibits elevated aFGF levels in affected tissues and may respond favorably to FGF antagonists (22). FGF antagonists may also be useful in attenuating herpes virus infection since it has recently been shown that HSV-1 uses the FGF receptor, flg, as a portal of entry into susceptible cells (23). This list highlights only a few of the potential therapeutic applications of FGFs and FGF antagonists and many more can be imagined (24).

In order to facilitate the development of FGF antagonists, we have chosen to clone and overexpress two distinct FGF receptors, flg and bek (25,26). This was necessary because, although most cell types express FGF receptors, their specific identification as particular gene products was unknown. In addition, their low expression (< 5000 receptors per cell) coupled with the high affinity of the FGFs for heparin sulfate proteoglycans found on most cells led to high background in binding assays.

Receptor Tyrosine Kinases

The cloning of full length cDNAs for human flg and bek has been described in detail (27). The FGF receptors are receptor linked tyrosine kinases which have signal transduction characteristics (28) very similar to the EGF and PDGF receptors which activate phospholipase C-γ as part of their signal transduction pathway (29-31). In addition, the earlier studies of EGF and PDGF receptors serve as valuable paradigms for our present characterization of the FGF receptors.

Receptor linked tyrosine kinases can be characterized into four broad families according to their structure (Table I). The first receptor tyrosine kinase (RTK) family, represented by the EGF receptor, consists of an extracellular domain containing two cysteine rich regions, a single transmembrane region and a cytoplasmic tyrosine kinase domain. The insulin receptor family (RTK II) are heterotetrameric structures

consisting of two identical heterodimers. Each heterodimer contains an extracellular chain containing two cysteine rich regions disulfide bonded to another chain which has a single transmembrane and a cytoplasmic tyrosine kinase. The RTK III family, represented by the PDGF receptor, contains five Ig-like domains in its extracellular region, along with a single transmembrane sequence, and a cytoplasmic tyrosine kinase containing an insert of 66-104 amino acids in the middle of the kinase domain. Each member of each family has an absolute requirement for tyrosine kinase activity in order to be biologically functional in signal transduction. In addition, all the RTKs appear to utilize receptor dimerization as a mechanism of receptor activation. Preliminary experiments indicate that these rules will hold for the FGF receptors as well.

The FGF receptors comprise a fourth RTK family which contains 3 Ig-like domains in its extracellular region with an acidic region located between the first and second Ig-like domains (27,32,33). The cytoplasmic tyrosine kinase domain is interrupted by a kinase insert similar to those of the PDGF receptor family. However, the kinase inserts of the FGF receptor family consist of only 14 amino aids as opposed to the much larger inserts of the RTK III family.

TABLE I

RECEPTOR TYROSINE KINASE FAMILIES

Family	Receptor Tyrosine Kinases
RTK I	EGF-R, HER2/neu HER3/c-erbB-3, Xmrk
RTK II	Insulin-R, IGF-1-R, IRR
RTK III	PDGF-R-A, PDGF-R-B, CSF-1, c-kit, flt
RTK IV	flg, bek, CEK2

The classification is adapted from Ullrich and Schlessinger (32) and is extended by the addition of CEK-2 (42) and flt (43).

The FGF receptors exhibit heterogeneity in their ligand binding domains. Although flg and bek were initially isolated as forms containing 3 Ig-like domains (27,33,34), several groups have characterized flg and bek forms which are missing the first Ig-like

domain, but which are proficient in binding (35-37). Direct comparison between forms containing either 2 or 3 Ig-like domains indicates that, at least for aFGF and bFGF, binding affinity is equivalent between the short and long forms (36). In addition, cDNAs coding for soluble, secreted forms of extracellular domains of flg and bek have been described (36,38) and may be important in regulating the FGF binding or signaling in vivo. A similar secreted form of the EGF receptor extracellular domain has been reported but no specific function for its presence has yet been elucidated (39).

The number of FGFs, together with multiple FGF receptors and various forms of the receptors, indicates that the biological interactions of FGFs and their receptors will probably be quite complex. Nevertheless, it is conceivable that a receptor antagonist which exhibits target specificity can be developed through intelligent drug screening coupled with a knowledge of FGF ligand and receptor biology.

Overexpression and Binding of the FGF Receptors

We generated the FGF receptor overexpressing cell lines, NFlg26 and NBek8, by transfecting NIH 3T3 cells with flg and bek expression vectors, respectively. NFlg26 cells express a predominant flg protein of 150 kDa with minor hyper- and hypo-glycosylated species of 170 kDa and 130 kDa. NBek8 cells synthesize a major bek species of 135 kDa. Treatment of NFlg26 and NBek8 cells with tunicamycin to prevent glycosylation results in a 100 kDa flg product and a 90 kDa bek product which are more consistent with the predicted primary translation sizes of 89 kDa. It has been proposed that glycosylation is necessary for binding of the FGF by the receptor (40). It should be easier to more rigorously address this question with site directed mutagenesis and overexpression now that the receptors have been cloned.

The NFlg26 and NBek8 cells express high levels of specific binding for FGFs as determined by equilibrium binding experiments. NFlg26 and NBek8 cells express a single class of high affinity sites for aFGF, bFGF, and hst/KFGF (27,28). The results of our binding data are summarized in Table II. Both flg and bek exhibit high affinity binding towards aFGF and bFGF (25-80 pM) and bek shows high affinity for hst/KFGF (80 pM). However, flg exhibits less affinity for hst/KFGF (~320 pM) than the other receptor/FGF interactions. Interestingly, a similar 15-fold difference in affinity between bFGF and hst/KFGF for a flg protein lacking the first Ig domain was observed by others (37). We have

extended these observations by showing that a bek deletion form, lacking the first Ig-like domain and acidic region, binds aFGF and bFGF with essentially equal affinity as full length bek (36). We can infer from these results that the determinants of binding specificity are contained within the second and third Ig-like domains.

TABLE II

BINDING OF OVEREXPRESSED FGF RECEPTORS

		Apparent Kds (pM)		
Cell Line	Receptors/Cell	aFGF	bFGF	hst/KFGF
NIH 3T3	5,000	60	ND	ND
NNeo4	5,000	60	ND	ND
NBek8	100,000	50	80	80
NFlg26	125,000	25	50	320

Generation of the flg and bek transfected 3T3 cells, NFlg26 and NBek8, and the control neomycin resistant NNeo4 cells is described in reference 27. The apparent dissociation constants were obtained by Scatchard analysis of equilibrium binding data (27,28).

Binding of the three FGFs to either flg or bek results in pronounced activation of the phospholipase C-γ signal transduction system as measured by tyrosine phosphorylation of the receptor and phospholipase C-γ and generation of inositol tri-phosphates (28). These results with cloned, overexpressed receptors, confirm the earlier conclusions that FGF signal transduction proceeds through tyrosine phosphorylation and activation of phospholipase C-γ (41).

CONCLUSIONS

In order to better understand the biology of the FGFs and to develop FGF antagonists, we have cloned and separately overexpressed two full length human FGF receptors, flg and bek. They comprise a separate receptor linked tyrosine kinase family which contains at least one other FGF receptor, CEK2 (42). It is likely that the family will grow as our knowledge of the present members extends our ability to search for other FGF receptors. Two of the receptors bind at least three ligands, which indicates a very high level of redundancy in FGF receptor/ligand interactions. The biological relevance of this redundancy will only be

appreciated once the expression patterns and relative affinities of the different components are known. We are presently cloning and expressing other FGFs and FGF receptors in order to complete our picture of relative affinities and are also collecting data on expression in potential therapeutic areas.

The cloning of FGFs and FGF receptors has been critical to our development of FGF antagonists. The overexpressing cell lines allow us to assess binding characteristics of specific identified gene products rather than the potentially mixed FGF receptor populations present on most cells. By removing certain domains, we have been able to determine that the binding activity of bek is contained within the second and third Ig-like domains (36). Further deletions and site specific mutagenesis should help to determine the minimum binding domains on both the ligands and receptors, thus aiding the design of potential antagonists.

From a practical point of view, the much greater signal to noise ratio obtained with these cells lines allows us to perform much more sensitive antagonist screening assays with either adherent cells or solubilized membranes. The FGF receptors in solubilized membranes exhibit similar pharmacology as the FGF receptors in cell monolayers. However, the filter binding assays with solubilized membranes are much more economical in terms of labor and cost of supplies and offer the best choice for primary screens of drug candidates.

Finally, the overexpressing cell lines serve as valuable reagents for the generation and screening of monoclonal antibodies, which may be potentially useful as antagonists in themselves, or at least useful for demonstrating the actual therapeutic utility of small molecule FGF receptor antagonists.

The strategies that we have employed in our work on FGF receptors should be generally applicable to other receptors which are potential drug targets. Naturally, the receptor antagonists which evolve from this program must be tested in appropriate animal models. The pleiotrophic effects of the FGFs, along with the high redundancy in the FGF system, offer very challenging opportunities in drug design.

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Discussion - FGF RECEPTORS AS TARGETS FOR DRUG DEVELOPMENT

A.J.H. Gearing

Can you review the data that supports the oligomerization model for signal transduction?

C. Dionne

We have data concerning the FGFs and the PDGFs receptors. If we take ^{125}I labeled acidic FGF and cross link it to cells we do not see dimerization, but if we do that same experiment and add DSS as a cross-linking agent we see the dimers. This has been done not only in just over-expressing cell lines but with soluble extracellular secreted forms. So it works independently of the cell membrane as well.

M.M. Reidenberg

Would you comment on where you see the selectivity in this system in the context of future drug development?

C. Dionne

That is why we are looking at the different binding domains of FGF receptors. The biggest problem is that these receptors are everywhere. Potential points of antagonist development are the kinase domain as well, but these are probably the least selective. We only have minor selectivity differences with HST binding to flg. Flg is expressed almost everywhere we look, and so is bek, although at much lower levels, except in embryonic cells where the expression is much higher. We are looking at the CEK2 gene, which is another FGF receptor, and that I think will be much more limited.
So we are at the very early stages.

R.G. Werner

You mentioned that the FGF has a whole range of activities, including angiogenesis and promotion of growth. If these compounds were used for wound healing, how would you see the problem of tumor promotion in treated patients?

C. Dionne

This is a very interesting point. The tumorigenicity only applies to cells that have set up an autocrine type system. One cannot get tumor formation in whole animals by injecting them with FGFs. However, cells that have been transfected and express their

own FGFs are chronically stimulated in a different way than chronic addition and tumorigenesis does then occur.

A.J.H. Gearing

Can you give us an idea of the levels of FGFs in say a healing wound or in an RA synovial fluid?

C. Dionne

The estimates are around ng/ml. This is one of the reasons why we are looking at antagonists rather than at agonists, although its exogenous addition does work. However, FGF 5 is everywhere and at relatively higher levels.