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Inhibition of the Type I Insulin-like Growth Factor Receptor Expression and Signaling: Novel Strategies for Antimetastatic Therapy

Pnina Brodt, Amir Samani and Roya Navab*

Departments of Surgery and Medicine, McGill University Health Center, Montreal, Quebec, Canada

ABSTRACT. The receptor for the type 1 insulin-like growth factor (IGF-1R) plays a critical role in the acquisition of the malignant phenotype. Using a highly metastatic murine lung carcinoma model, it was demonstrated that this receptor regulates several cellular functions that can impact on the metastatic potential of the cells, including cellular proliferation, anchorage-independent growth, cell migration, and invasion. The tumor model was used to develop several strategies for altering receptor expression and function as means of abrogating the metastatic potential of the cells. They include stable expression in the tumor cells of IGF-1R antisense RNA and dominant negative receptor mutants in which tyrosines in the kinase domain were substituted with phenylalanine. In addition, a novel strategy was used based on altering post ligand-binding receptor turnover. This led to inhibition of receptor re-expression and signaling and resulted in increased tumor cell apoptosis. When combined with the development of viral vectors designed to deliver genetic information with high efficiency, these strategies could form the basis for development of highly specific, antimetastatic therapy in tumors with known IGF-IR involvement. BIOCHEM PHARMACOL **60**;8:1101–1107, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. IGF-I receptor; cancer metastasis; signal transduction

The ability of cancer cells to metastasize and form cancerous lesions in secondary sites still poses the most formidable obstacle to cancer cure. Metastatic disease unresponsive to chemotherapy, surgery, and radiotherapy is fatal because it interferes with the functions of affected viscera such as liver, lung, bone, and brain. Lung and liver metastases, which are frequently associated with late-stage disease in malignancies such as breast and prostate carcinomas, carcinomas of the gastrointestinal tract, osteosarcoma, and melanoma, are generally refractory to common chemotherapeutic drugs and often inaccessible to surgical resection [1]. Therapeutic treatment of metastases in these vital organs will therefore require the development of innovative, biology-based approaches that can harness novel information on the molecular biology of metastasis to identify molecular targets and develop pharmaceutical reagents that can interfere with their expression and/or functions.

The establishment of a metastasis is the final outcome of a dynamic process that involves multiple interactions between the disseminating cancer cells and their rapidly changing microenvironments. It involves cell detachment from the primary tumor, migration, and invasion of host tissue barriers and intravasation. Once in the circulation, tumor cells may adhere to the vascular endothelium through specific receptors, extravasate into the target organ interstitium and parenchyma, and utilize both autocrine and paracrine growth regulatory mechanisms to grow in the secondary site. The newly formed lesions can themselves become the source of disseminating cells that repeat this cycle and give rise to tertiary metastases [2–4, see schematic representation in Fig. 1]. These multiple processes are mediated by a series of molecular interactions resulting from the deregulated expression and/or function of cell–cell and cell–extracellular matrix (ECM) adhesion receptors, ECM degrading proteinases, growth promoting factors, and their receptors [3]. Disruption of these molecular interactions at any one of these steps could potentially lead to abrogation of the entire process.

THE RECEPTOR FOR TYPE 1 INSULIN-LIKE GROWTH FACTOR (IGF-IR)

One family of molecules critical for malignant transformation and metastasis are the peptide growth factors that regulate cell entry into and progression through the cell cycle by binding to membrane receptor tyrosine kinases (RTK), which transmit signals to the nucleus through an intricate network of adaptor and signaling molecules [5–7]. One of the RTKs implicated in the induction and maintenance of the transformed/malignant phenotype is the re-

* Corresponding author: Dr. Pnina Brodt, Dept. of Surgery, Division of Surgical Research, McGill University Health Center, Royal Victoria Hospital, Room H6.25, 687 Pine Ave W., Montreal, Quebec, Canada H3A 1A1. Tel. +1-514-842-1231; FAX +1-514-843-1411; E-mail: pnina.brodt@muhc.mcgill.ca.

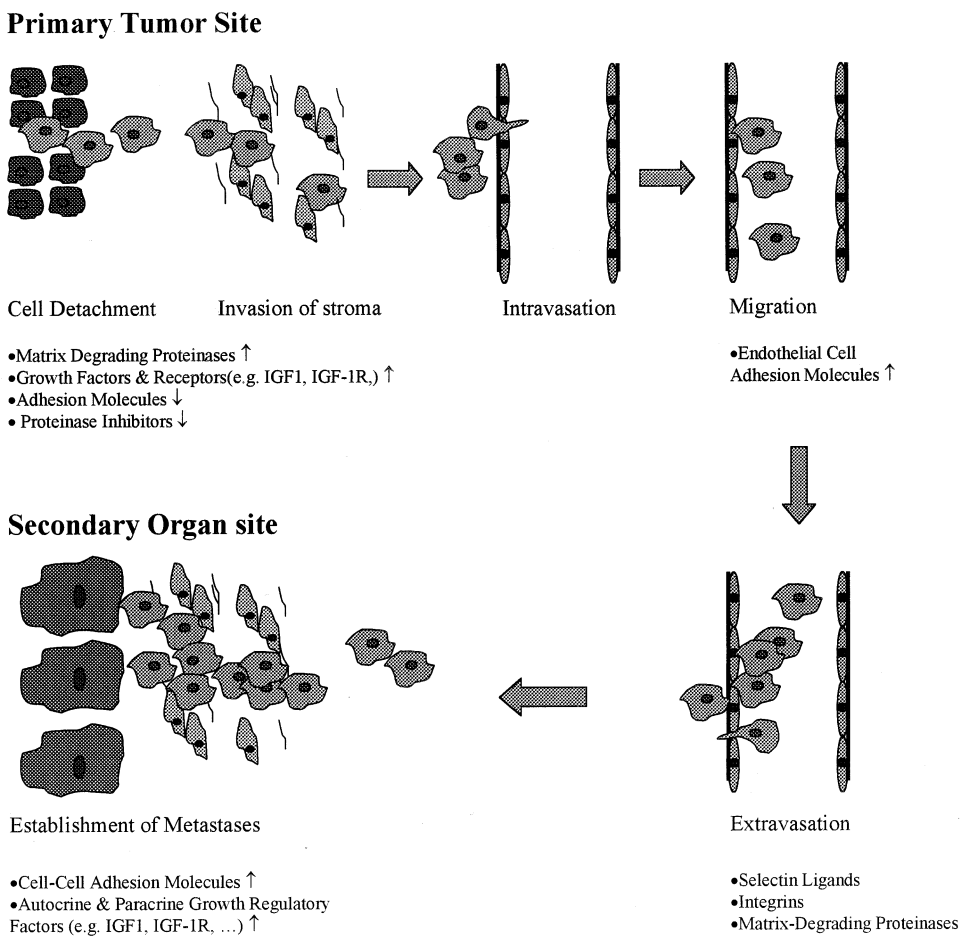

FIG. 1. The basic steps in cancer metastasis. Some of the molecular mechanisms and mediators known to be involved or altered at each of the steps in the metastatic cascade are indicated.

ceptor for the IGF-IR.* This heterotetrameric receptor consists of two 130–135 kDa α- and two 90–95 kDa β-chains, with several α–α and α–β disulfide bridges [8, 9]. The ligand-binding domain is located on the extracellular α-subunit. Approximately one-third of the β-subunit is extracellular and is connected to the intracellular portion by a single transmembrane domain. The intracellular region of the β-subunit has a binding site for phosphorylation substrates at tyrosine residue 950, an ATP-binding site at lysine 1003, a tyrosine kinase domain with 3 critical tyrosines at positions 1131, 1135, and 1136, and several tyrosines in the carboxyl domain at positions 1250, 1251, and 1316, all of which have been implicated in the regulation of the receptor's biological functions (Fig. 2). The IGF-IR ligands include IGF-I, IGF-II, and insulin, but it binds IGF-I, a 70-amino acid peptide with the highest affinity [10]. Binding and physiological activities of IGF-I can be modulated by its association with the IGF-binding proteins (IGFBPs), a family of structurally related, secreted proteins that bind both IGF-I and IGF-II with high affinities and regulate their biological accessibility and activity [11, 12].

The IGF-I receptor plays an important role in the regulation of the cell cycle [13]. During postnatal development and longitudinal growth, growth hormone functions are mediated via IGF-I. IGF-I serum levels are high during childhood, declining progressively after puberty. IGF-IR mRNA levels also decline considerably after puberty, remaining high in selected tissues such as the brain and kidney [10]. Malignant transformation, however, is often associated with up-regulated expression and/or constitutive activation of the IGF-IR [14]. A requirement for IGF-I for cell survival and growth has been documented in a broad range of cell types where it is thought to act in concert with initiation factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) and mediate cell cycle progression from G_1 to S phase [13, 15, 16]. In addition to and distinct from its role as a positive regulator of cell growth, IGF-I is also a survival factor and could block apoptosis in a range of cell types *in vitro* [17, 18] and *in vivo* [19].

SIGNAL TRANSDUCTION BY THE IGF-IR

In response to ligand binding, the intrinsic tyrosine kinase of the receptor is activated, resulting in autophosphoryla-

* *Abbreviations:* IGF-1R, receptor for the type I insulin-like growth factor; IRS, insulin receptor substrate; and PI-3K, phosphatidylinositide-3 kinase.

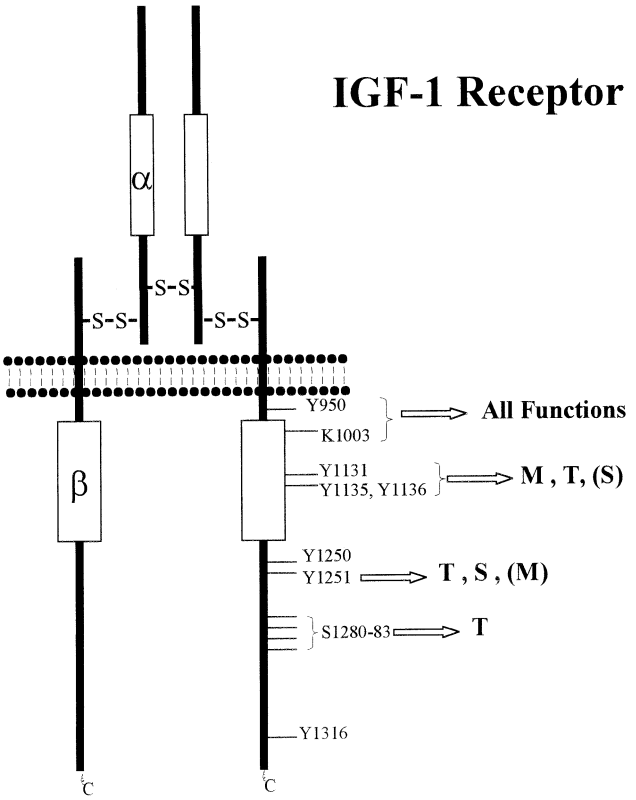

FIG. 2. Structure of the IGF-I receptor. Amino acid residues involved in signal transduction are shown. Their postulated roles in receptor-mediated functions based on mutational analyses are indicated on the right. M-mitogenic response, S-survival, T-transformation.

tion of tyrosines on the intracellular portion of the β-subunit and the subsequent tyrosine phosphorylation of several downstream substrates including the IRS 1–4 and Shc [20, 21]. The IRS constitute a family of structurally related adaptor proteins that can link the IGF-I receptor to downstream signal transduction mediators regulating cellular growth. Of these, IRS-1 is the most extensively studied [22]. This 165–195-kDa molecule does not contain SH2 (Src homology 2) or SH3 domains and may bind to the β-subunit through a PTB (pTyr-binding) domain [22, 23]. It contains at least 20 potential tyrosine phosphorylation sites and can act as a multisite "docking" protein associating with multiple downstream signaling proteins including PI-3 kinase [24], SH2 domain-containing tyrosine phosphatase (Syp) [25], Fyn, Nck, and growth factor receptor-bound protein-2 (Grb2) through their SH2 domains [26]. Stimulation of PI-3K leads to activation of several downstream substrates including protein kinase B (Akt), which can phosphorylate BAD and attenuate its proapoptotoic effect and the pp70 S6 kinase [20, 21]. Grb2 is tightly associated with the guanine nucleotide exchange factor mSOS linking the IGF-1R to the Ras/Raf-1/ mitogen-activated protein kinase (MAPK) signaling pathway, leading to activation of nuclear transcription factors [14, 27, see Fig. 3]. Like IRS-1, tyrosine phosphorylation of Shc promotes association with Grb2, linking it to the Ras pathway via the Grb2–mSOS complex [7]. Preferential phosphorylation of Shc or IRS-1 could depend on the cellular context and may direct IGF-IR signaling preferentially towards cellular proliferation or differentiation [28]. In some cell types, the IGF-IR can also directly phosphorylate the Janus kinases (JAK) 1 and 2 involved in cytokine-mediated signaling and JAK proteins may in turn phosphorylate IRS-1 [29]. Phosphorylation of JAK proteins can lead to phosphorylation/ activation of signal transducers and activators of transcription (STAT) proteins, in particular STAT-3 [30], and STAT-3 activation may in turn be essential for the transforming activity of IGF-1R [31].

Other direct substrates of IGF-IR include the proto-oncogenes c-Crk II and CrkL and the p125 focal adhesion kinase (FAK) [32–34]. These molecules can link IGF-IR to integrin-mediated signaling and the cytoskeleton through p130(Cas) and paxillin, and the regulation of cell shape and motility [34, 35]. Because intracellular calcium levels increase in response to IGF-1 binding, PLCγ is also thought to be involved through its products inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) [14]. The relative importance of these pathways in signal transduction by IGF-IR is probably cell context-dependent and remains to be fully elucidated.

Mutational analyses have identified several domains in the receptor β-subunit containing amino acid residues essential for receptor functions. Lysine at position 1003 (the ATP-binding site) and tyrosine 950 (thought to be essential for IRS-1 and Shc binding and phosphorylation) are critical for all receptor functions [23, 36, 37]. Tyrosines 1131, 1135, and 1136 in the kinase domain are essential for the mitogenic and transforming activities of the receptor [38, 39], but there is some controversy regarding their role in regulating the antiapoptotic effect of the receptor [40]. In contrast, the tyrosines in position 1251, although not essential for receptor, IRS-1, and Shc phosphorylation, are critical for the transforming (anchorage-independent growth) and antiapoptotic effects of the receptor, possibly through involvement in regulating cytoskeletal reorganization [40–42]. The role of these tyrosines in mitogenesis is controversial [41, 42]. Finally carboxyl-terminal serines in positions 1280–1283 also appear to play a role in regulating the transforming function of the receptor [43, see Fig. 2].

ROLE OF IGF-1R IN MALIGNANCY

Several lines of evidence implicate IGF-1 and the receptor in malignant progression. Increased expression of IGF-I, IGF-IR, or both has been documented in many human malignancies including carcinomas of the lung, breast, thyroid, gastrointestinal tract and prostate, glioblastomas, neuroblastomas, rhabdomyosarcomas, and leukemias [reviewed in 44]. Furthermore, prospective clinical studies identified high plasma IGF-I levels as a potential risk factor for carcinomas of the breast, prostate, and colon [45–47]. In addition, the IGFs are potent mitogens for a wide range of tumor cell types *in vitro*. Several oncogenes have now been

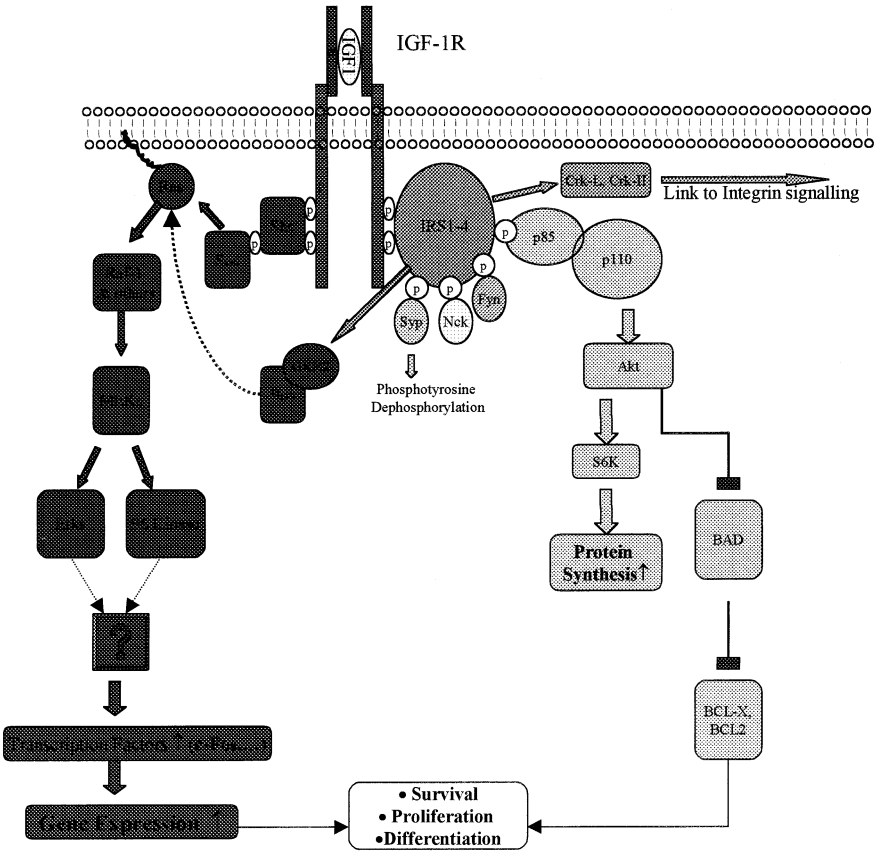

FIG. 3. Ligand binding-induced signal transduction by the IGF-I receptor. Ligand binding triggers IGF-I receptor autophosphorylation at multiple tyrosine residues. The intrinsic receptor tyrosine kinase then phosphorylates multiple substrates including IRS 1–4 and Shc. The IRS proteins act as docking sites for multiple proteins including PI-3K which can provide cell survival signals through activation of Akt. Shc and IRS phosphorylation links the receptor to the Grb-2/mSOS complex and the Ras/Raf-1/MAPK signaling cascade, leading to changes in gene transcription.

shown to affect IGF-I and IGF-IR expression [13]. Suppression of IGF-IR by antisense oligonucleotides [48], plasmids expressing IGF-IR antisense cDNA [49, 50], IGF-I peptide analogues [51], and triple-helix-forming oligodeoxynucleotides [52] suppressed tumor cell growth *in vivo* [reviewed in 53, 54].

ROLE OF IGF-IR IN METASTASIS

Our laboratory has been studying molecular factors that regulate tumor cell dissemination using a mouse tumor model of Lewis lung carcinoma sublines with distinct metastatic properties. IGF-IR was identified as a positive regulator of the invasive/metastatic phenotype and IGF-I as a paracrine growth-promoting factor in the liver [55, 56]. Highly metastatic carcinoma H-59 cells express high IGF-IR levels, and these were found to be critical for tumor cell ability to form metastases in the liver. H-59 cells expressing IGF-1R antisense RNA, lost mitogenic and motogenic responses to IGF-I, had reduced levels of the extracellular matrix-degrading metalloproteinase MMP-2, became non-invasive, and failed to form metastases in the liver following intrasplenic/portal inoculation [57, 58]. These studies identified IGF-IR as a potential biological target for antimetastatic therapy and are the basis of ongoing efforts to develop additional antimetastatic strategies based on suppression of IGF-IR expression and function.

STRATEGIES FOR SUPPRESSION OF IGF-IR EXPRESSION AND FUNCTION

We investigated two additional approaches for suppression of IGF-IR expression and function: 1. Inhibition of signaling through the use of receptor dominant negative mutants and 2. Blocking receptor trafficking by inhibition of endosomal endopeptidases.

Use of Receptor Dominant Negative Mutants

Dominant negative mutants of the IGF-I receptor have been successfully used by other laboratories to block IGF-IR functions [59–60]. To investigate the potential use of dominant negative receptors to block tumor invasion and metastasis, we recently used site-directed mutagenesis to generate domain-specific mutants of the IGF-1 receptor β-subunit. Highly invasive IGF-IR$^+$ H-59 cells were transfected with a plasmid vector expressing full-length human

IGF-1 receptor cDNA in which the codons for tyrosines Y1131, Y1135, and Y1136 in the kinase domain were substituted with codons for phenylalanine. Stably transfected cells were analyzed with respect to changes in invasive and metastatic ability relative to unmodified cells or cells transfected with the wild-type receptor. The kinase domain mutant receptor had a dominant negative effect and significantly reduced the invasive and metastatic potential of these cells.* The results demonstrated that IGF-IR kinase domain mutants can block the metastatic potential of tumor cells *in vivo*, suggesting that dominant negative mutant receptors could provide an alternative or complementary approach to antisense-based reagents designed to reduce the number of functional receptors available on the cell surface. In a clinical setting, where micrometastases have either been diagnosed or are suspected, the successful use of such reagents will depend on the availability of highly efficient vehicles for delivery of genetic material into the tumor cells. Because normal liver parenchymal cells do not constitutively express measurable levels of IGF-IR and receptor levels are up-regulated in several malignancies with known hepatic involvement (e.g. colorectal and breast carcinomas), liver metastases may be particularly suited for IGF-IR-directed therapy based on the delivery of genetic information such as antisense or dominant negative receptors designed to alter receptor levels and/or functions.

Inhibitors of Receptor Trafficking

An alternative strategy for suppression of IGF-IR signaling is the targeting of post ligand binding events that regulate receptor turnover [36]. Like other peptide growth factors, binding of the IGF-1 to its receptor triggers receptor-mediated internalization of the ligand. This process is thought to be a key event in the regulation of receptor bioavailability and activity [62, 63]. IGF-I dissociates from its receptor within the acidic environment of the endosomes, a process that may be mediated by intraendosomal cysteine proteinases such as cathepsins B and L and is thought to be required for receptor recycling to the cell surface [62, 64–66]. Abrogation of ligand dissociation is known to lead to the intraendosomal accumulation of ligand and receptor and to disruption of receptor functions [63, 66]. Because this strategy only affects cycling cells, it is expected to have minimal deleterious effects on the majority of the normal, non-dividing hepatic cells, and therefore target cancer cells with high specificity. Recent reports including our own suggest that cysteine proteinase inhibitors can indeed alter tumor cell growth and IGF-IR signaling [67, 68].

* Brodt P, Ling H, Fallavollita L, Khatib AM and Zhang D, manuscript in preparation.

CONCLUSIONS

The IGF-I receptor has been identified as a critical mediator of malignant transformation and a positive regulator of the metastatic phenotype. In recent years, it has emerged as a potential biological target for antimetastatic therapy. Several strategies for abrogation of IGF-IR expression and signaling have been tested in our model. They include manipulation of gene expression by antisense RNA, the use of dominant negative receptor mutants to alter receptor function, and inhibition of receptor–ligand processing to block receptor re-expression and signaling. Recent advances in the development of viral vectors for highly efficient delivery of genetic information into cells should provide the tools for translation of these experimental approaches into clinically relevant therapeutic modalities for the treatment of metastatic disease.

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