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Growth factor receptors: targets for gene therapy and immunotherapy for cancer treatment

Review Article

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Abbreviations: angiopoietin 1, (Ang 1); angiopoietin 2, (Ang 2); epidermal growth factor, (EGF); fibroblast growth factor, (FGF); Fibroblast growth factor receptors, (FGFR); platelet-derived growth factor, (PDGF); tyrosine kinase activity, (RTK); vascular endothelial growth factor, (VEGF); Vascular endothelial growth factor receptors, (VEGFR)

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Summary

Angiogenesis has a crucial role in the invasion, growth and metastasis of solid tumors. Known angiogenic growth factors derived from tumors and vascular cells include VEGF, FGF, Ang-1, Ang-2, EGF, IGF and PDGF. Angiogenic growth factors bind to receptors with tyrosine kinase activity (RTK). These RTKs are overexpressed or mutated in a variety of human cancers, therefore they represent a potential target for anti-cancer therapy. Recent advances in the fields of gene therapy and molecular biology can be applied to design new vaccines and antitumor therapies for the treatment of cancer. In this review, the use of RTKs for anti-angiogenic immunotherapy and anti-angiogenic gene therapy is described, followed by a comparison among several gene delivery methods.

I. Introduction

Angiogenesis is the development of new capillaries from preexisting blood vessels in normal and malignant tissues; it occurs in a variety of conditions including embryonic development, wound healing and tumor growth. Extensive studies have established that angiogenesis has a central role in the invasion, growth and metastasis of solid tumors (Eberhard et al, 2000) (Folkman, 1996; Goede et al, 1998). Although tumors less than 2 mm in diameter can receive nutrients and remove waste products by diffusion, further growth depends on persistent angiogenesis (Folkman, 1990). Known angiogenic growth factors, derived from tumors and vascular cells, include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoietin 1 (Ang 1) and 2 (Ang 2), epidermal growth factor (EGF), insulin-like growth factor and platelet-derived growth factor (PDGF). Tumor-associated macrophages have been shown to secrete a wide variety of angiogenic factors including IL-8, VEGF, TGF-α and TGF-β (Sunderkotter et al, 1994). Endothelial cells adjacent to a tumor can produce FGF, TGF-β and PDGF (Saltis et al, 1992).

Angiogenic growth factors are extracellular signals that regulate endothelial cell proliferation and differentiation; most of them bind to receptors with RTK. The RTKs are monomers, located in the cell membrane. Ligand binding in the extracellular domain induces receptor dimerization and trans-phosphorylation of specific tyrosine residues, located in the cytoplasmic portion of the receptor (Brunelleschi et al, 2002). Phosphorylation of these RTKs induces activation, resulting in RTK-mediated signal transduction and endothelial cell proliferation. Over-expression or mutation of RTKs have been found in human cancers, therefore, they represent a potential target for cancer therapy. Several approaches have been used to block the binding between the RTKs and their growth factor ligands: antibodies against the growth factor receptor, tyrosine kinase inhibitors and soluble forms of receptors, which are able to sequester the growth factor by attenuation or by blocking the downstream signal transduction that leads to angiogenic responses. This review will focus on the use of some RTKs as potential vaccines and gene therapy-based targets for treating cancer. To distinguish the two different approaches, the following definitions are given:

The use of RTK as a vaccine to induce RTK-specific immune cells or antibodies to inhibit angiogenesis is referred to as RTK anti-angiogenic immunotherapy. Treatment with a dominant-negative form of RTK *in vivo* to directly inhibit endogenous RTK, thus inhibiting angiogenesis, is referred to as RTK anti-angiogenic gene therapy.

RTK anti-angiogenic immunotherapy and gene therapy are attractive approaches for treating cancer because: 1) they can provide common targets to treat multiple malignancies; 2) endothelial cells are genetically stable, which avoids the concern of antigen-loss, as often occurs in tumor cells; 3) it is possible to combine RTK anti-angiogenic immunotherapy with tumor antigen-based immunotherapy to further improve antitumor efficacy.

With the identification of specific biological mediators of angiogenesis, it is now possible to administer these molecules to inhibit the angiogenesis process. Progress in gene therapy and molecular biology has provided technology to find new tumor antigens and also to deliver these tumor antigens in an effective way to induce immune responses in cancer patients.

II. Vascular endothelial growth factor receptors (VEGFR)

VEGF stimulates neovascularization in a variety of tumors; elevated VEGF levels are often measured in sera from cancer patients (Tuttle et al, 2002). VEGF exerts its biological effects on endothelial cells by binding to its cell surface receptors. There are 3 receptors: VEGFR-1(Flt-1), VEGFR-2(Flk-1) and VEGFR-3(Flt-4). They are all RTKs and have seven Ig-like domains in the extracellular portion, a transmembrane region and an intracellular tyrosine kinase region (Schlessinger and Ullrich, 1992). VEGF can interact with VEGFR-1 and VEGFR-2 but not with VEGFR-3. VEGF-B binds to VEGFR-1, VEGFC and VEGFD bind to VEGFR-2 and VEGFR-3. Cultured primary endothelial cells are able to express VEGFR-1, 2 and 3, making it difficult to evaluate the contribution of each individual receptor regulated by each individual VEGF (Kaipainen et al, 1995). VEGFR-1 binds VEGF with a 10-fold higher affinity than VEGFR-2 (Terman et al, 1992), however, VEGFR-2 is the major receptor, transducing the effects of VEGF into endothelial cells. Binding of VEGF to the receptors induces tyrosine phosphorylation of the intracellular domain, leading to activation of intracellular signaling and subsequent phenotypic changes, such as endothelial survival, proliferation and migration (Kliche and Waltenberger, 2001). VEGFR-1 and VEGFR-2 have been used as angiogenesis inhibitors to sequester VEGF, in order to attenuate the further steps of angiogenesis.

Ye et al, (2004) constructed a retroviral vector expressing a soluble VEGFR-1 gene. Transfer of soluble VEGFR-1 gene effectively inhibited the growth of xenografted human FTC-133 thyroid tumors by 70% in immunodeficient mice. Takayama et al, (2000) constructed an adenovirus, expressing the extracellular domain of the human VEGFR-1 fused to the Fc portion of human IgG. When human lung cancer cells infected with Ad-VEGFR-1 were injected into nude mice, the tumors stopped growing around day 10 after inoculation and decreased in size thereafter. Because Ad-VEGFR-1 infection did not affect cancer cell growth *in vitro*, the authors assumed that the inhibitory effect on tumor formation was presumably due to indirect mechanisms, such as inhibition of tumor angiogenesis. These two groups showed tumor inhibition, using VEGFR-1 for RTK anti-angiogenic gene therapy. Niethammer et al, (2002) immunized mice with an orally administered DNA vaccine encoding murine VEGFR-2, carried by attenuated *Salmonella typhimurium.* Mice were protected from melanoma, colon carcinoma and lung carcinoma cells. The VEGFR-2 DNA vaccine was also effective in a therapeutic setting in the mice, which were vaccinated 10 days after injection of colon carcinoma cells when they had fully established pulmonary metastases. They showed the involvement of $CD8^+$T cells in the antitumor immune response, given that *in vivo* depletion of $CD8^+$T cells, before the challenge with tumor cells, resulted in extensive growth and dissemination of pulmonary metastasis. Other approaches, such as reducing the VEGF level or inhibiting endothelial cells, also demonstrated great potential for inhibition of tumor growth.

Another example used by Abdollahi et al, (2003) is to use endostatin, a fragment of collagen XVIII that inhibits endothelial proliferation and a VEGFR-2 antagonist. They inoculated nude mice with human prostate adenocarcinoma and human glioblastoma cell lines and waited for the tumors to establish. Administration of endostatin plus VEGFR-2 antagonist resulted in a significantly greater tumor growth delay, than the one produced by either therapy, alone. The combination of decreasing endothelial proliferation, plus the inhibition of angiogenesis, by blocking the interaction between VEGF andVEGFR-2 may increase the therapeutic effectiveness of antitumor activity.

III. Fibroblast growth factor receptors (FGFR)

FGF constitute a family of heparin-binding polypeptide mitogens. The well characterized family members are FGF-1 and FGF-2, formerly known as acidic and basic FGF. Four receptors have been identified in the human (FGFR-1, FGFR-2, FGFR-3 and FGFR-4); they have an extracellular ligand-binding domain containing 3 Ig-like loops, a transmembrane domain and a cytoplasmic region that contains the tyrosine kinase catalytic domain (Dionne et al, 1990; Partanen et al, 1990).

Hughes, (1997) studied the tissue distribution of all four receptors in humans. The most widespread expression was FGFR-1 and FGFR-2. High levels of FGFR-1 were seen in the skin, cornea, lung, heart, placenta, kidney and ureter. Abundant FGFR-2 expression was found in the prostate and stomach. FGFR-3 expression was seen in the appendix, colon, liver, sublingual gland, placenta and cervix. FGFR-4 expression was found in the liver, sublingual gland ducts, kidney, ureter and some arterioles and veins. Endothelial cells in the angiogenic vessels around tumors express FGF-1, FGF-2 and high affinity receptors FGFR-1 and FGFR-2 (Carmeliet and Jain, 2000).

He et al, (2003) constructed two vaccines of plasmid DNA with *Xenopus* FGFR-1 and mouse FGFR-1. BALB/c mice were immunized with plasmid DNA encoding FGFR-1 and were subsequently challenged with Meth A fibrosarcoma, H22 hepatoma or MA 782/5S mammary

carcinoma cells. Only *Xenopus* FGFR-1 but not mouse FGFR-1 vaccination inhibit tumour growth. The mice depleted of $CD4^+$ T cells did not develop detectable antibodies against FGFR-1 and were not protected from tumor challenge, whereas treatment with anti-CD8 or anti-NK monoclonal antibodies failed to abrogate the anti-tumor activity. By using a gene involved in angiogenesis from a different species, this group was able to raise a $CD4^+$ antibody-dependent immune response. These data correlate with the result from Wei et al, (2001), who immunized mice with plasmid DNA encoding *Xenopus* VEGF or murine VEGF. They also found that only *Xenopus* VEGF (xenogeneic gene) was protective and the antitumor response was $CD4^+$ T lymphocyte-dependent.

IV. VEGFR and FGFR combinations

Considering that VEGF and FGF are both potent angiogenic factors, some groups tried a combination of soluble receptors to improve the anti-tumor efficacy in different types of cancer.

Ogawa et al, (2002) prepared a soluble VEGFR-1 adenoviral vector and a soluble FGFR-1 adenoviral vector for RTK anti-angiogenic gene therapy. The combined administration of Ad-VEGFR-1 and Ad-FGFR1 produced an enhanced suppression of tumor growth for human pancreas cancer and human lung cancer cells in nude mice. These results suggest that VEGFs and FGFs make independent contributions to tumor angiogenesis, producing a synergistic anti-tumor effect.

Kanda et al, (2004) recently proposed that signals *via* VEGFR-1 but not VEGFR-2, are necessary for FGF-2-induced capillary morphogenesis, one of the steps to angiogenesis, by endothelial cells. This result suggests that VEGFR-1 and VEGFR-2 are involved in different pathways toward angiogenesis and therefore, simultaneously blocking both receptors VEGFR-1 and VEGFR-2 would inhibit angiogenesis more efficiently to than blocking only one.

V. Tie-2

Both Ang-1 and Ang-2 have been identified as ligands for Tie-2, a receptor expressed on endothelial cells. Binding of Ang-1 to Tie-2 induces tyrosine phosphorylation and maintains and stabilizes mature vessels by promoting interactions between endothelial cells and the surrounding extra-cellular matrix. Ang-2 competitively binds to Tie-2 and antagonizes the stabilizing ability of Ang-1, resulting in an overall destabilization of vessels (Wojchowski et al, 1999). Ahmad et al, (2001) reported that the expression of Ang-2 but not of Ang-1 was strongly upregulated during carcinogenesis, indicating that the switch in the balance between Ang-1 and Ang-2 may play an important role in tumor angiogenesis and growth.

Hawighorst et al, (2002) showed that overexpression of angiopoietin-1 in squamous cell carcinoma A431 enhanced Tie-2 receptor phosphorylation *in vivo* and resulted in a significant inhibition of tumor growth (70%). In addition, there was an increase in the percentage of mature blood vessels corresponding to the induction of Tie-2 phosphorylation *in vivo*. Overexpression of Ang-2 did not modulate Tie-2 phosphorylation and accordingly, failed to affect tumor growth and angiogenesis.

Nair et al, (2003) immunized C57BL/6 mice 3 times with DC cells pulsed with Tie-2 mRNA. Eight days after the last immunization, mice were challenged with B16/F10.9 melanoma tumor cells. RTK anti-angiogenic immunotherapy with Tie-2 resulted in tumor inhibition in mice. Since Tie-2 is expressed in proliferating endothelial cells but not in B16/F10.9 tumor cells, the observed tumor inhibition was an indirect consequence of interfering with the tumor neovascularization process.

VI. The EGFR family

The EGF receptor family is comprised of four homologous receptors: ErbB1(HER-1) (EGFR), ErbB2 or HER-2/neu, Erb3 or HER-3 and ErbB4 or HER-4. These receptors are composed of an extracellular binding domain, a transmembrane lipophilic segment and an intracellular protein tyrosine kinase domain with a regulatory carboxyl terminal segment (Klapper et al, 2000). At least six different ligands, known as EGF-like ligands, bind to the ErbB1. EGF-like ligands induce formation of ErbB1/ErbB1 homodimers and ErbB1/ErbB2 heterodimers. Elevated expression of both ErbB receptors and their ligands is detected in several types of human cancers, for example breast, lung, head and neck, colorectal, ovarian and prostate (Salomon et al, 1995).

Lu et al, (2003) immunized mice with human or murine extracellular domain EGF receptor (ErbB1) and evaluated the protective and therapeutic effect in Lewis lung cancer and mammary cancer. Protection and a significantly greater survival only existed in the mice immunized with human ErbB1. They found that *in vivo* depletion of $CD4^+$ T lymphocytes could completely abrogate the antitumor activity with the immunization of human ErbB1, whereas depletion of $CD8^+$ T lymphocytes showed partial abrogation of the antitumor activity *in vivo*. These data correlate with data from He et al, (2003) who also found that $CD4^+$ T cells was responsible for protecting mice immunized with *Xenopus* FGFR1 plasmid DNA, as described in section III.

Lachman et al, (2001) showed an 80 % protection for mice vaccinated with HER-2/neu and challenged with A2L2 breast tumor cells. They also immunized mice with a lower dose of plasmid DNA; this experiment resulted in a 40% protection anainst A2l2 breast tumor cells. They observed a Th1 response confirmed by IFN-γ production.

Wei et al, (1999) compared the vaccination efficacy of the transmembrane and cytoplasmic ErbB-2. They found that full-length and functionally active tyrosine kinase neu was the most protective vaccine against D2F2 mammary tumors expressing neu, however, the potential of HER-2/neu to become an oncogene in patients is a major safety concern. All of the mice immunized with the cytoplasmic form of ErbB-2, either with or without the tyrosine kinase mutation, developed tumors. The same group (Pilon et al, 2001) performed another study, in which the cytoplasmic form of ErbB-2 was combined with GM-CSF and IL-2; the full-length mutant tyrosine kinase and the cytoplasmic mutant tyrosine kinase were also

combined. All of these combinations were significantly protective against tumor incidence, compared to the control. The rejection of D2F2 mammary tumors expressing neu was Ab-independent and $CD8^+$T cell-dependent. The combination of secreted ErbB-2 plus cytoplasmic ErbB-2 was 100% protective against tumor incidence, providing both Th1 and Th2 responses (Piechocki et al, 2001). These results showed that RTK anti-angiogenic immunotherapy, with DNA encoding proteins in different locations (extracellular vs intracellular), can change the protection level of the vaccine because of the stability of the recombinant protein *in vivo* and the predominance of diverse types of immune responses (antibody, Th1, Th2).

VII. Insulin-like growth factor receptor (IGFR)

IGF-I and IGF-II have been shown to be strong mitogens for a wide variety of cancer cell lines including prostate, breast, colon, myeloma, melanoma, ovary and lung. This mitogenic effect is mediated through the IGF-I receptor, a tyrosine kinase receptor that closely resembles the insulin receptor in structure and signaling cascades.

Two RTK anti-angiogenic gene therapy studies have reported the use of IGF-IR to treat established lung and ovarian tumors in nude mice. Lee et al, (2003) constructed two recombinant adenoviruses expressing IGF-IR with an engineered stop codon, one at position 950 and the other at position 482. These 2 molecules produce defective truncated receptors that lack the tyrosine kinase domain and compete for ligands in the extracellular environment. Lung cancer xenografts, established in nude mice, were treated with two recombinant adenoviruses using intratumoral injection. Adenovirus IGF-IR/482 showed significant inhibition of human lung tumor growth, compared to the control. Hongo et al, (2003) administered a soluble IGF-IR 486 amino acid recombinant protein, partially purified in CaOV-3 human ovarian tumors, established in nude mice. Treatment with IGF-IR significantly retarded tumor formation of CaOV-3 cells in nude mice, for 6 weeks. Tumors began to grow when they stopped the protein administration. These two groups independently showed that a truncated, soluble form of IGF-IR is a good candidate to treat lung and ovarian cancer patients.

VIII. Platelet-derived growth factor receptor (PDGFR)

PDGF is a potent mitogen for mesenchymal cells including fibroblasts, smooth muscle cells and glial cells. PDGF also induces other diverse and important cellular processes including chemotaxis, survival, apoptosis and transformation *in vitro* (Deuel et al, 1982). There are 4 genes of PDGF (A, B, C and D) that form homodimers or heterodimers. PDGF isoforms exert their cellular effects by activating two related cell surface receptor tyrosine kinases (α-PDGFR and β-PDGFR). The three dimeric PDGF receptors (αα, ββ, αβ) mediate PDGF isoform-specific signal transduction (Yu et al, 2003). Most malignant mesothelioma cell lines express β-PDGFR, whereas normal mesothelioma cells predominantly express α-PDGFR. PDGF, released by tumor cells, induces migration of endothelial cells and vascular smooth muscle cells, and also stimulates proliferation of these cells, suggesting a direct role of PDGFs in angiogenesis. Several studies indicate the significance of PDGF in human tumors, including glioma, dermatofibrosarcoma, neurofibroma, myelomonocytic leukemia, osteoblastoma and osteosarcoma (Hermanson et al, 1992; Kadono et al, 2000; Sulzbacher et al, 2000).

Lokker et al, (2002) studied the expression of PDGF ligands and receptors in 11 human glioblastoma tumor cells. The expression of PDGF/PDGFR pairs that are known to functionally interact were identified in all the samples. Therapeutic treatment of nude mice with a PDGFR antagonist reduced xenograft tumor growth by 44% after subcutaneous injection of C6 glioblastoma cells. The fact that β-PDGFR induces only positive signaling for cell transformation, makes it a strong candidate to be used for RTK anti-angiogenic gene therapy. α-PDGFR is also expressed in several tumors, however it plays a role in both positive and negative signals for cell transformation.

IX. Delivery systems

Antitumor molecules used for either vaccination or for gene therapy, can be delivered as attenuated microorganisms (adenovirus, *Salmonella*, *Listeria*), cells (dendritic cells, tumor cells), proteins or naked DNA. Gene therapy and DNA vaccines offer many advantages over peptides or attenuated pathogens; DNA is very stable and easy to prepare in large quantities. DNA vaccines have been shown to be an effective therapy against cancer in animal models, although the mechanisms responsible for the generation of an immune response by DNA immunization are still not well-understood. In addition, plasmid DNA could potentially integrate into the host genome, leading to malignant transformation. Efficiency of transfection is dependent on the efficiency of DNA delivery and the efficiency of DNA expression. Since a critical step for successful vaccination or gene therapy is to deliver DNA into the cells *in vivo*, methods for enhancing DNA cell penetration have been widely explored. There are mechanical methods, such as gene gun and jet injection, electrical methods (electroporation) and chemical methods, based on the complex formation between positively charged chemicals (polymers) and negatively charged DNA molecules. Trimble et al, (2003) showed higher $CD8^+$ T cell responses and antitumor efficacy by E7/HSP70 DNA vaccine administered through gene gun or biojector, compared to needle intramuscular injection. In addition, DNA vaccination *via* gene gun-based DNA delivery required the lowest dosage to generate similar antitumor effects compared to that of needle intramuscular injection. and biojector. *In vivo* Electroporation gene transfer has been proven to be efficient for gene delivery in muscle, skin, liver and tumors (Aihara and Miyazaki, 1998; Rols et al, 1998; Suzuki et al, 1998). Electroporation can dramatically reduce the amount of DNA necessary to raise an immune response, by increasing DNA uptake and gene expression.

Electroporation has been effective in treating murine squamous cell carcinoma in mice, with intratumoral or intramuscular electrotransfer of IFN-α (Li et al, 2001) and IL-12 (Li et al, 2002). Using the electroporation technique to enhance anticancer drug delivery to tumor cells is called electrochemotherapy and is already being applied clinically against head and neck cancers (Heller et al, 1999). Therefore, electroporation may be useful for increasing the effectiveness of DNA vaccines in humans.

X. Conclusions

Recent advances in gene therapy and molecular biology have made it possible to improve the technology to treat tumors *in vivo*. The RTK anti-angiogenic immunotherapy and gene therapy are very promising, although there are still some important issues that need to be investigated. Many RTK gene therapies have shown antitumor efficacy in several types of cancers because they can inhibit or reduce angiogenesis. However, a primary concern of immunizing against angiogenesis-associated products is interference with normal angiogenesis. The potential adverse effects of RTK anti-angiogenic immunotherapy and gene therapy needs to be explored in future experiments.

The delivery system used for vaccination is crucial for the activation of T cell response. Cells and attenuated pathogens have been shown to be effective for vaccination, however, it is difficult to produce them in large-scale and they need special conditions to survive for a long period of time. Plasmid DNA, on the other hand, is easy to produce and very stable, even at room temperature. Electroporation can help to reduce the amount of DNA necessary for vaccination or gene therapy, therefore it contributes to reduction of the risk of toxicity. Further knowledge is needed to determine the best conditions for use of this technology in humans. There are other issues to consider for successful vaccination and gene therapy, that still need to be explored further, for example, the location of the protein in the host (extracellular vs. intracellular) and the use of syngeneic vs xenogeneic molecules. The location of the protein in the host can determine the activation of antibodies, $CD4^+$ or $CD8^+$ T lymphocytes and the use of xenogeneic molecules can break the immune tolerance to self proteins in the host. In conclusion, RTK anti-angiogenic immunotherapy and gene therapy have been successful in the mouse model and may provide a valuable tool for cancer treatment in humans.

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