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REVIEW

Intrabodies: turning the humoral immune system outside in for intracellular immunization

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Antibodies have long been used in biomedical science as in vitro tools for the identification, purification and functional manipulation of target antigens; they have been exploited in vivo for diagnostic and therapeutic applications as well. Recent advances in antibody engineering have now allowed the genes encoding antibodies to be manipulated so that the antigen binding domain can be expressed intracellularly. The specific and high-affinity binding properties of antibodies, combined with their ability to be stably expressed in precise intracellular locations inside mammalian cells, has provided a powerful new family of molecules for gene therapy applications. These intracellular antibodies are termed 'intrabodies'. Two clinical protocols have been approved by the RAC for the use of intrabodies in the treatment of an oncologic and an infectious disease. Their clinical use will in all likelihood become widespread if these initial studies show 'proof in principle'. In this article, the studies from laboratories that have used intrabodies as molecular reagents for cancer therapy and for the control of infectious diseases will be reviewed and future directions of this technology will be discussed.

Keywords: *antibody engineering; intracellular antibodies; gene therapy; AIDS; oncogenes*

Introduction

The humoral immune system is incredibly diverse in its ability to produce antibodies to virtually any target molecule whether it be self or foreign, protein or nucleic acid, carbohydrate or lipid. Immunologic tolerance normally prevents the development of autoantibodies, however, numerous disease states in humans provide evidence that tolerance can be broken. Once these *in vivo* control mechanisms are removed, the development of human antibodies to virtually any molecule of interest, whether of human or microbial origin, should be readily achieved. Indeed recently, a powerful new set of immunologic tools has been developed that has allowed human antibody genes to be easily manipulated.[1–3] Using these tools, the creation of large human immunoglobulin libraries from naive individuals has been achieved and when combined with phage display technology, has allowed investigators to bypass *in vivo* immunization and produce high-affinity human antibodies to human proteins.[4–7] Smaller immunoglobulin libraries from individuals with autoimmune[8,9] or infectious diseases.[1,10,11] have allowed more disease-specific antibodies to be readily isolated. Transgenic mice that contain a human immunoglobulin locus in the absence of the corresponding mouse locus have been produced and stable hybridomas that secrete human antigen-specific antibodies have been reported.[12,13] Thus, although almost all of the intrabodies thus far reported have used well characterized and affinity matured rodent hybridoma cell lines as starting materials for the isolation of the specific antibody genes of interest, these powerful new *in vitro* methods described above, when combined with the ability to express these antibody molecules inside the cell, provide a rich source of human antibody genes and form the basis of an extraordinarily powerful new family of protein molecules that have potential applications in the gene therapy of a number of human diseases.[14–16]

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Intrabody design

By harvesting the power and diversity of the immune system in the form of rearranged antibody genes, the immune system itself accomplishes the binding site design, thereby bypassing some of the technical hurdles involved in RNA-based intracellular immunization strategies that use antisense, ribozymes and RNA decoys. A key factor contributing to the success of recent studies has been the use of single-chain antibodies, also known as single-chain variable region fragments or sFv, in which the heavy- and light-chain variable domains are synthesized as a single polypeptide and are separated by a flexible linker peptide, generally $(Gly_4\text{-}Ser)_3$. The constant portion of the immunoglobulin molecule, which has no role in antigen binding, is dispensed of entirely. The result is a small (approximately 28 kDa) molecule with high-affinity ligand-binding capability and minimal assembly requirements. The addition of a human constant domain to the carboxy-terminus of the sFv has increased the activity of several of the anti-HIV-1 intrabodies discussed below.[17] Other examples of Fab (monovalent antigen binding fragment) intrabodies have been reported,[18,19] but only where an internal ribosomal

entry site has been used to allow stoichiometric amounts of Fd heavy and light chains to be expressed simultaneously is a practical application for their use likely;[20,21] bulk transductions with vectors containing separate promoters to drive heavy and light chain expression have in our hands resulted in greater variability in the co-expression of the two chains. The intrabodies can be directed to the relevant cellular compartments by modifying the intrabody genes with N-terminus or C-terminus polypeptide extensions that encode classical intracellular-trafficking signals.[14–16] Functional studies have illustrated that both sFv and Fab intrabodies are able to fold and assemble correctly in the cytoplasm, even though: (1) the chaperones that would normally assist in this process are localized in the endoplasmic reticulum (ER) and, therefore, unavailable; and (2) the reducing environment does not favor the formation of disulfide bonds. However, it has been generally found that cytosolic intrabodies (whether they have intact chains, Fab or sFv fragments) exhibit shorter half-lives than their secretory counterparts.[22]

Gene therapy applications of intrabodies

In principle, intrabodies can be used to modulate cellular physiology and metabolism by a wide variety of mechanisms. For example, binding by an intrabody may be used to:

- block or stabilize macromolecular (eg protein–protein or protein–DNA interactions;
- modulate enzyme function by occluding an active site, sequestering substrate, or fixing the enzyme in an active (on) or inactive (off) conformation;
- divert proteins from their usual cellular compartment, for example, by sequestering transcription factors in the cytoplasm, or by retention in the ER of proteins that are destined for the cell surface or to be secreted.

Down-regulation of growth-factor receptors

Intrabodies that are targeted to the lumen of the ER provide a simple and effective mechanism for inhibiting the transport of plasma membrane or secreted proteins to the cell surface; even highly abundant cell-surface receptors have been reduced to undetectable levels using this method. Intrabodies that are intended for localization in the ER are generally equipped with a leader peptide and a C-terminus ER retention signal (the KDEL amino acid motif – Lys-Asp-Glu-Leu). In practice, the KDEL tag is not always necessary since a number of sFv intrabodies designed for secretion appear to be export incompetent and remain in the ER for reasons that are, at present, unclear.[23–25]

A KDEL-tagged sFv intrabody has been used to down-regulate the α subunit of the receptor for human interleukin 2 (IL-2Rα, Tac, CD25).[25] IL-2Rα plays a key role in T cell-mediated immune responses, and is constitutively overexpressed in some T and B cell leukemias, most notably in adult T cell leukemia (ATL) which is caused by HTLV-1. The specific induction of IL-2Rα on all HTLV-1-transformed cells, including fresh ATL cells, has led to the hypothesis that IL-2 receptor signaling may be involved in the process of HTLV-1 leukemogenesis. An ER-targeted sFv derivative of the anti-Tac monoclonal antibody was found to completely abrogate the cell surface expression of IL-2Rα in PMA-stimulated Jurkat cells.[25] IL-2Rα was detectable within the Jurkat cells as a 40 kDa precursor form that was sensitive to endoglycosidase-H, consistent with its retention in a pre-Golgi compartment. The sFv intrabody was used to down-regulate IL-2Rα in several (IL-2-independent) HTLV-1-transformed cell lines which normally express high levels (approximately 200 000 molecules per cell) of IL-2Rα. IL-2Rα expression was reduced to undetectable levels without affecting cell viability or growth rate and therefore was not required for the growth of the cell lines that were examined.[26] Intrabodies against IL-2Rβ and γ_c, which play a more direct role in IL-2 signal transduction, can be used in future studies to examine the role of IL-2 receptor signaling in the process of HTLV-1 leukemogenesis. Although these intrabodies may offer significant potential for immunomodulation and for the control of IL-2R-dependent tumor cell growth *in vivo*, especially when used in combination with targeted gene delivery systems, further work will be required to validate these hypotheses.

Targeted tumor killing via anti-erbB2 sFv intrabodies

Two groups have reported the use of ER-targeted sFv intrabodies to inhibit expression of the erbB2 transmembrane protein. ErbB2 is a member of the type I/epidermal growth factor receptor (EGFR)-related family of receptor tyrosine kinases which include erbB/EGFR, erbB2, erbB3 and erbB4. ErbB2 becomes rapidly phosphorylated and activated following ligand treatment of many cell lines. These naturally occurring ligands such as epidermal growth factor (EGF) and Neu differentiation factor (NDF) do not bind directly to erbB2, and its activation is likely to be mediated via transmodulation by other members of the type I/EGF-related family of receptor tyrosine kinases (RTKs).[27] ErbB2 is amplified or overexpressed in a variety of human tumors, including breast and ovarian carcinoma, where it correlates with an unfavorable prognosis.[28–31]

Two KDEL-tagged sFv intrabodies have been shown to decrease markedly the cell-surface expression of erbB2 in NIH3T3 fibroblasts that express an oncogenically activated form of erbB2. Functional inactivation of the receptor was suggested by a reduction in the phosphotyrosine content of the cells and, more importantly, by the reversion of the cells to a non-transformed phenotype.[32] One of these KDEL-tagged sFv intrabodies was further studied in T47D human mammary carcinoma cells that express four members of the type I/epidermal growth factor receptor family including erbB/EGFR, erbB2, erbB3 and erbB4. Analysis of signals elicited by ligands of EGFR, erbB3 and erbB4 demonstrated that in cells stably expressing the anti-erbB2 KDEL-tagged sFv intrabody and selectively lacking cell surface erbB2, phosphorylation of Shc, activation of MAPK and $p70/p85^{S6K}$, and induction of c-*fos* expression in response to both EGF and NDF were impaired.[27] These studies also show the importance of receptor transmodulation among the type I RTKs.

In complementary studies, an ER-directed sFv intrabody has been used to down-regulate erbB2 in the human ovarian-carcinoma cell line SKOV3. Transient expression

of the intrabody gene in SKOV3 cells led to the loss of erbB2 expression at the cell surface. Furthermore, it was difficult to establish SKOV3 clones that stably expressed the anti-erbB2 sFv intrabody, suggesting that constitutive down-regulation of erbB2 is incompatible with the long-term survival of these cells.[33] When *ex vivo*-transduced SKOV3 cells were introduced subcutaneously into nude mice, no tumor grew and complete tumor eradication at necropsy 80 days after s.c. transplant occurred only in mice receiving the anti-erbB2 expressing cells in marked contrast to mice receiving the control transduced cells.[34] Tumor cell eradication in transduced primary cultures of human ovarian carcinoma cells could also be demonstrated.[34] Using a replication-defective adenovirus to *in vivo* transduce the anti-erbB2 sFv intrabody gene into i.p. transplanted SKOV3 cells, these investigators further demonstrated *in vivo* tumor cell killing and a significantly prolonged survival of animals compared with control groups.[35] Although issues related to *in vivo* gene delivery and nonspecific toxicity still exist,[36] based on these and other preclinical data, a clinical gene therapy protocol to treat metastatic ovarian cell carcinoma in patients that have failed conventional chemotherapy was approved by the United States Recombinant DNA Advisory Committee (RAC) in 1995.

Inactivation of cytosolic oncoproteins

Several groups of investigators have constructed intrabodies, derived from the parental hybridoma Y13–259, to perturb the function of $p21^{ras}$, a guanine nucleotide-binding protein that is strategically involved in the control of cell growth and differentiation.[37–41] Members of the *ras* gene family have been implicated in many types of human cancer. Intrabody expression in the cytosol has generally been accomplished by simple removal of the immunoglobulin leader sequences. In two studies, microinjection of mRNA encoding an anti-$p21^{ras}$ sFv intrabody was microinjected into *Xenopus* oocytes and was shown to inhibit insulin-induced meiotic maturation of the cell, a process known to be $p21^{ras}$-dependent.[37,38] Meiotic maturation was not inhibited in oocytes that co-expressed cytosolic forms of the separate heavy and light chains, presumably reflecting the more stringent assembly requirements of a native four-chain antibody. In other studies in *Xenopus* oocytes, activation of p42 mitogen-activated protein kinase (MAPK) by *ras* was also strongly inhibited by the sFv intrabody.[40] Two groups have transfected either sFv intrabodies[40] or whole immunoglobulin heavy and light chains (in separate expression plasmids)[41] into morphologically transformed cells with activated *ras* and have demonstrated phenotypic reversion to a non-transformed morphology. All of these data suggest anti-*ras* intrabodies may have a role in cancer gene therapy in the future.

Cyclin D1 overexpression contributes to the transformed phenotype of numerous cancerous cell lines. Preliminary data have been presented on the inhibitory effect of an anti-human D1 sFv intrabody in stably transfected MCF7 breast cancer cell lines.[42] Intrabodies that interfere with the function of cytosolic kinases, GTPases or other molecules involved in signal transduction could prove extremely valuable in unravelling the complex and interconnecting pathways that serve to deliver extracellular signals to the nucleus.

Inhibition of virus replication

Intrabodies have important therapeutic potential in the defense of microbial pathogens. One study has demonstrated inhibition of infectious tick-borne flavivirus production by cytosolic sFv intrabodies directed against a cross-reactive epitope on the envelope protein of louping ill and some other tick-borne encephalitis viruses.[43] In another study, inhibition of HTLV-1 Tax-mediated transactivation of the HTLV-1 LTR was demonstrated.[44] However, perhaps no better example of the broad use of intrabodies for the gene therapy of a human infectious disease comes from studies on the human immunodeficiency virus, HIV-1. Indeed, sFv and Fab intrabodies have been used to prevent HIV-1 replication by blocking both post-integration and pre-integration steps in the virus life cycle. Post-integration inhibition of HIV-1 replication has been achieved by blocking of gp120 processing and incorporation into virions.[19,21,25,45,46] Transduction of a human anti-gp120 sFv intrabody gene into PBMCs from both uninfected and HIV-1-infected patients has been reported using murine leukemia virus (MuLV) vectors that encoded the sFv intrabody gene under the control of an internal CMV promoter.[45,46] In HIV-1 challenge experiments, both cytoprotection and inhibition of HIV-1 replication were observed. Based on these encouraging results, a clinical gene therapy protocol was approved by the RAC in 1995. This study will evaluate the safety and efficacy of intracellular antibody gene therapy in asymptomatic patients with HIV-1 infection by reinfusing autologous $CD4^+$ T cells that have been transduced *ex vivo* with a MuLV vector that expresses the anti-gp120 sFv intrabody. In addition to following various immunologic and viral parameters, the *in vivo* kinetics and survival of intrabody-transduced cells will be compared by limiting dilution PCR with those of a separate aliquot of cells transduced with a control vector (identical except for the intrabody gene).

Post-integration blockage of HIV-1 replication by inhibiting critical HIV-1 regulatory protein functions such as Tat-mediated viral transcriptional transactivation,[45,47] or Rev-mediated nuclear export of singly spliced or genomic viral RNA[48–50] has also been reported. Potent antiviral activity against a panel of primary HIV-1 isolates has been demonstrated. Intrabodies against these viral proteins are particularly attractive for clinical gene therapy trials because of the essential role of these proteins in the viral life cycle.

Pre-integration blockade of virus replication has been demonstrated with Fab intrabodies against the HIV-1 matrix protein (MA, p17)[21] and reverse transcriptase.[19] Intrabodies against other critical HIV-1 proteins are under study in a number of laboratories and the use of sFv intrabodies against different HIV-1 target proteins, when used in combination in bicistronic gene transfer vectors,[20,21] provides an approach that may prove more durable in suppressing virus replication than single intrabody therapy alone. Indeed, the recent success of pharmacologic-based studies that have compared combination agent therapy with single agent therapy lends support to this idea.[51–53] Intrabodies against cellular proteins that may prevent or negatively modulate HIV-1 replication will be an important area of research in the future. Candidate cellular proteins include the HIV-1 co-receptors[54–58]

and molecules that are involved in apoptosis of HIV-1-infected cells.[59–61]

Perspectives

The results obtained to date suggest that intrabodies represent a powerful alternative to other methods of gene inactivation, such as antisense RNA, dominant-negative mutants and targeted gene disruption. Unlike the dominant-negative approach, which can only be applied to certain types of protein, there are few theoretical constraints on the use of intrabodies. Indeed, intrabodies may represent the only option in circumstances where the desired target has not been cloned, or is non-protein in nature (eg sugar, DNA or a soluble metabolite). Owing to the diversity of the immunoglobulin V gene repertoire, the major hurdle in any given application may be that of identifying an antibody with the desired specificity and affinity; several studies have demonstrated that the epitope on the target molecule is critical to achieve the desired biological effect.[17,50] In fact, two reasons for the potent inhibitory activity of the intrabodies thus far reported may reside in the relative ease of directing the intrabodies to relevant subcellular compartments and to precise epitopes on the target protein.

Improving the stability of intrabodies that are expressed outside of the secretory compartment (eg in the cytosol) is one area that needs further development. While several studies have demonstrated that relatively short-lived intrabodies can achieve the desired biological effect, the power of the technology would undoubtably be enhanced by factors that reduce intrabody turnover and increase proper folding efficiency. Intrabodies also have the potential, not only to disrupt protein function, but to act in a positive fashion, for example, by enhancing protein activity, by stabilizing protein–protein or protein–nucleic acid interactions, or by performing catalytic functions themselves.

As in the case of other gene-based therapies, certain issues need to be addressed before intrabody technology can be widely applied in the clinical context. These include developing gene-transfer systems that are capable of delivering the intrabody gene into a sufficient number of the correct cell type; sustained and high enough expression of the intrabody gene to achieve the desired clinical benefit; and the intrabody protein should be non-toxic and non-immunogenic unless they are directed to oncoproteins where toxicity and immunogencity could contribute to their anti-tumor effect. While it is difficult to predict the success or failure of the clinical gene therapy trials that will be performed in the near future, it is clear that intrabody technology is in its infant stage and could find widespread application, both in basic science and molecular medicine.

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