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Targeting tumor cell destruction with CD64-directed bispecific fusion proteins

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Introduction

The human immunoglobulin G Fc receptor type I (FcγRI), or CD64, is a member of the immunoglobulin supergene family, and is the only FcγR with high affinity for monomeric IgG [5]. CD64 is expressed on a variety of leukocytes including monocytes, macrophages, dendritic cells, and $CD34^+$ myeloid progenitor cells. In addition, polymorphonuclear cells can be induced to express FcγRI with granulocyte-colony-stimulating factor and interferon γ (IFNγ) treatment, both in vitro and in vivo [23, 27]. CD64 mediates antibody-dependent cellular cytotoxicity (ADCC), phagocytosis, and release of inflammatory mediators [5]. The discrete expression of CD64 (confined primarily to cells with cytotoxic effector functions), and its potent immune triggering functions make this receptor an attractive target for redirected cytotoxicity.

Several antibodies have been developed that are specific for human CD64 [1, 12]. The monoclonal antibody (mAb) 22 binds to CD64 at a site distinct from the Fc ligand-binding domain, allowing binding of mAb 22 in the presence physiological concentrations of IgG [12]. mAb22 has been humanized to H22 through the process of complementarity determinant region grafting, to increase the potential use of this targeting antibody for clinical applications [10]. Several bispecific antibodies (bsAb) comprising mAb 22 (both the murine, M22, and humanized versions) and anti-target mAb have been constructed. The ADCC, phagocytosis, and superoxide generation mediated by these bsAb demonstrate that M22/H22 can trigger effector cell functions through CD64 [5, 10, 12, 25].

Several members of the epidermal growth factor receptor (EGF-R) family are associated with over-expression in specific malignancies, and are appropriate targets for tumor immunotherapy. In particular, EGF-R is over-expressed in almost all head and neck, kidney and lung tumors, and in about one-third of breast and ovarian tumors [20] and can be over-expressed in cancers of the prostate, bladder, brain, pancreas, and gastrointestinal system. The HER2/*neu* or erbB-2 protein is over-expressed in breast, ovarian, and other cancers [18, 24, 26]. The other members of the EGF-R family, HER3 (erbB-3) and HER4 (erbB-4) are less well characterized, but their expression has also been associated with carcinomas [15, 21].

Previous studies have shown that growth-factor-receptor-specific mAb can be successfully utilized for making chemically linked bispecific antibodies (bsAb) that target HER2/*neu*-expressing tumor cells to CD64-expressing effector cells [27, T. Keler et al. manuscript submitted]. We have developed genetically linked CD64-targeted bispecific fusion proteins employing the natural ligands of members of the EGF-R family [8]. CD3-directed cytotoxic fusion proteins with ligands for tumor-associated receptors as tumor-targeting reagents have been described previously [6, 17]. The natural ligand fusion proteins may be more advantageous than chemically linked bsAb by being easier to manufacture and having reduced immunogenicity. Furthermore, such fusion proteins may have direct growth-modulatory effects through interaction with the growth factor receptor. For example, the growth of tumor cell lines that over-express the EGF-R is inhibited by EGF [3, 14, 19]. This report summarizes our current work with two CD64-targeted fusion proteins, H22-EGF and H22-heregulin β (H22-HRG). The H22-EGF fusion protein binds specifically to cells that over-express EGF-R, while the H22-HRG is targeted to cells that overexpress HER2, HER3, and/or HER4.

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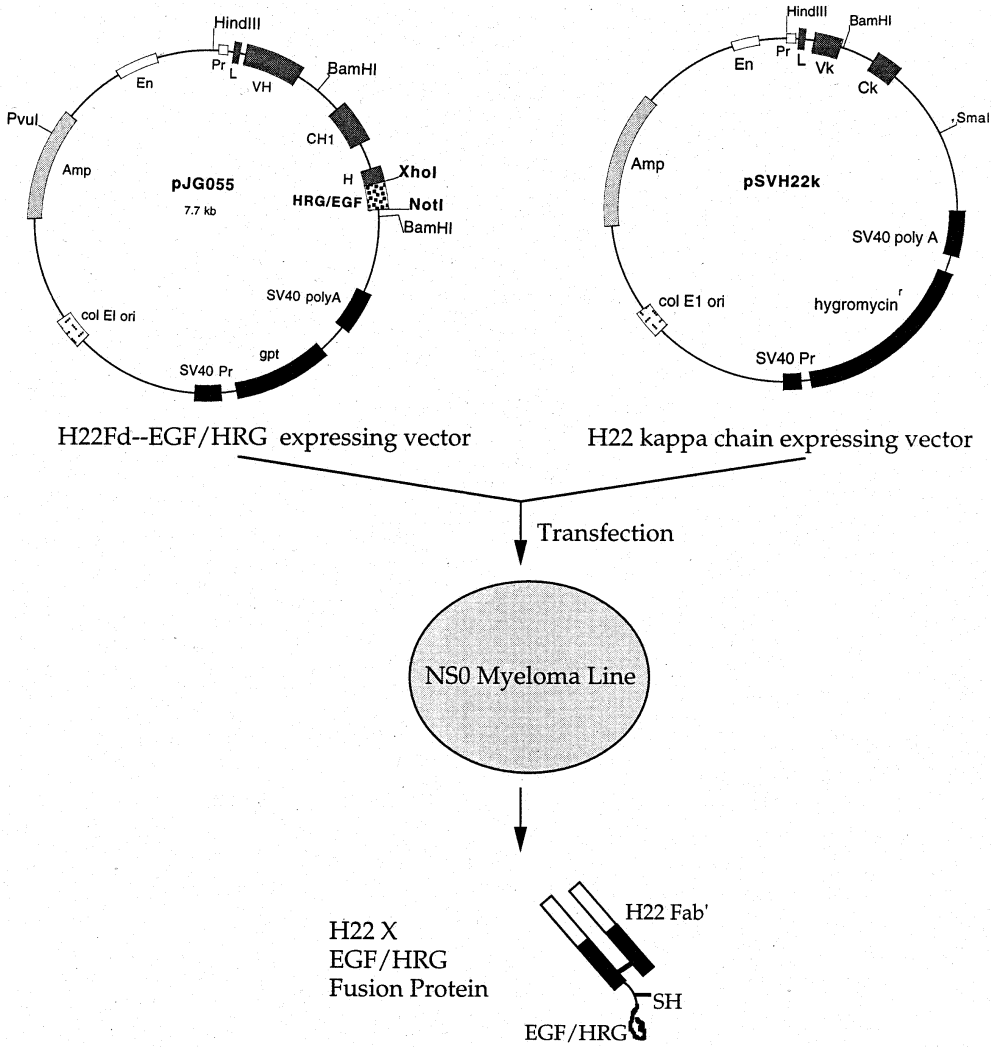

Fig. 1 Flow diagram showing the vectors used for transfecting NS0 cells resulting in the secretion of the H22-fusion proteins

Results and discussion

Genetic construction of bispecific fusion protein

Genomic DNA encoding the Fd fragment of humanized anti-FcγRI mAb, H22, was either fused to cDNA encoding human epidermal growth factor (EGF), resulting in a H22Fd-EGF-expressing vector, or was fused to cDNA encoding the EGF-like domain of the β-2 form of heregulin, resulting in H22Fd-HRG. These constructs were independently transfected into the NS0 myeloma cell line, which was previously transfected with a vector containing DNA encoding the H22 κ light chain (Fig. 1). The design that was used to generate the fusion protein construct consists of a translation-termination codon downstream of the cloning restriction sites, *Xho*I and *Not*I, and upstream of a *Bam*HI site, which was used to clone the new polymerase-chain-reaction-generated CH1 fragment downstream of VH. The cloning sites, which are located downstream of most of the hinge in order to retain flexibility between the Fd and ligand domains, was used to insert DNA encoding EGF or HRG. Also, a single Cys residue was retained to allow chemical linkage to other polypeptides using disulfide or thioether linkages. The chance of involving other FcγR in the immune-effector-cell-mediated biological activities of these fusion proteins is eliminated since the Fc portion of the H22 molecule has been removed. Therefore, binding to effector cells can only be mediated by the H22 V region binding to CD64.

Purification and characterization of the fusion proteins

These constructs were transfected into myeloma cells expressing the H22 light chain and the secreted product was purified by affinity chromatography as either a monomer H22F(ab′)-EGF or a H22F(ab′)-HRG molecule even though a cysteine remained in the hinge region [8]. This result is consistent with previously published reports where Fab′ molecules have been expressed both in mammalian and bacterial systems [4, 7, 22]. These fusion proteins inhibited the binding of labeled mAb 22 to CD64-expressing cells, indicating that they retained their ability to bind specifically to CD64 [8]. In addition, H22-EGF specifically bound EGF-R on tumor cells that overexpress this receptor [8]. H22-HRG bound to tumor cells expressing HER3/4 and

Table 1 Monocyte-mediated antibody-dependent cell cytotoxicity in conjunction with mAb H425 or with the H22-EGF fusion protein. Monocytes were prepared from a leukopak as previously described [12]. The monocytes were cultured for 24 h in the presence of 100 IRU interferon γ prior to being used as effector cells. Target cells were labeled with 100 μCi ^{51}Cr for 1 h at 37 °C. After washing, the targets were incubated with various concentrations of H22 F(ab′)2, H425, or H22-EGF in a U-bottomed micotiter plate with monocytes and incubated for 18 h, after which supernatants were removed and analyzed for radioactivity. Percentage lysis = (experimental ^{51}Cr release – target ÷ ^{51}Cr leak detergent lysis ^{51}Cr – target leak ^{51}Cr) ×100. Ab-dependent lysis = percentage lysis with antibody or fusion protein – percentage of lysis in medium only

Ab	Concentration (μg/ml)	Antibody-dependent lysis of A431 cells (%)	Antibody-dependent lysis of HN5 cells (%)
H22 $F(ab')_2$	0.01	1	1
H425	0.01	23	21
H22-EGF	0.01	28	53
H22 $F(ab')_2$	0.1	0	–1
H425	0.1	43	58
H22-EGF	0.1	47	58

its binding could be inhibited by β-heregulin but not by EGF, demonstrating its receptor specificity (data not shown).

H22-EGF mediated dose-dependent cytotoxicity of EGF-R-overexpressing cell lines. The data in Table 1 showed the relative ability of mAb H425, H22-EGF and the $F(ab')_2$ fragment of H22 to mediate dose-dependent lysis of A431 or HN5 cells in the presence of IFNγ-treated human monocytes. These results demonstrated that both H425 and the fusion protein were capable of killing EGF-R-over-expressing cells in the presence of FcγRI-expressing effector cells. The maximal level of ADCC mediated by the fusion protein was comparable to that mediated by the whole H425 antibody, although H22-EGF was more potent at lower concentrations. Likewise, the H22-HRG fusion protein was able to mediate lysis of the SKBr 3 tumor cell line, which expresses HER2/3/4 in the presence of FcγRI-expressing effector cells. Specific cytotoxicity ranged from 25% to 40% at concentrations ranging from 0.1 μg/ml to 1.0 μg/ml of H22-HRG fusion protein.

The ability of the fusion proteins to transmit signals via its ligand portion was demonstrated by the ability of the H22-EGF molecule to inhibit the growth of A431 cells (Table 2). Although EGF acts to stimulate growth of normal cells that express receptors for it, EGF can also act to inhibit growth of tumor cells that over-express EGF-R [3, 14, 19]. Both EGF and the H22-EGF fusion protein inhibited the growth of EGF-R-over-expressing tumor cell lines in a dose-dependent fashion ([8], Table 2). mAb H425, which binds EGF-R at or near the EGF-binding site, required significantly higher concentrations to inhibit cell growth. These results demonstrated that the H22-EGF molecule was able to transmit biochemical signals via both the H22 and EGF portions.

Among the many results of HRG signaling observed in tumor cells is the up-regulation of ICAM 1 expression [2]. ICAM 1 is an adhesion molecule that is a ligand for CD11a and CD11b, molecules that are expressed by immune

Table 2 EGF and H22-EGF inhibit the growth of A431 cells. A431 cells (2×10^4) were added to six-well plates in medium alone or in medium containing various concentrations of mAb H425, EGF, or H22-EGF. Viable cells were counted 8 days later using a hemocytometer. Results are reported as percentage growth inhibition as compared to cells grown in media alone

Inhibitor	Concentration (nM)	Growth inhibition (%)
mAb H425	0.1	0
	1.0	14
	10.0	38
EGF	0.1	68
	1.0	98
	10.0	98
H22-EGF	0.1	30
	1.0	93
	10.0	97

effector cells of both lymphoid and myeloid lineages. We have demonstrated that the treatment of SKBr 3 cells with H22-HRG induced ICAM 1 levels in a dose- and time-dependent fashion that was comparable to induction by β-HRG. ICAM 1 levels were enhanced as much as tenfold upon treatment with H22-HRG compared to control-treated cells, as determined by binding of an ICAM-1-specific mAb. Since myeloid-cell-mediated ADCC has been shown to require LFA-1/ICAM interaction [11], the increased level of ICAM 1 induced by H22-HRG may result in augmented myeloid cell-mediated ADCC of tumor cells in vitro and in vivo.

Because there is one free cysteine residue retained in the hinge region, heterodimeric fusion proteins may be produced using H22-EGF chemically conjugated to H22-HRG creating a molecule containing three specificities. The antitumor effects of one fusion protein monomer may therefore be enhanced to target two different receptors over-expressed on the same or different cancer cells using such a trispecific molecule. Furthermore, the design of our genetic construction lends itself to the development of other fusion proteins. The *Xho*I and *Not*I restriction sites in the hinge region of the H22 heavy-chain expression vector may be used to clone any cDNA directionally that has been engineered to contain these restriction sites. For example, H22-CD4 fusion protein may be used to target HIV or HIV-infected cells that express gp120.

This technology can also be applied to enhancing the immune response to a given antigen. Gosselin et al. [9] have demonstrated that antigen-specific T cell responses to tetanus toxoid could be augmented by directing the antigen to FcR on monocytes and macrophages by chemically conjugating the antigen to anti-FcR-specific mAb. This concept has been demonstrated in vivo using CD64 transgenic mice [13]. The cDNA encoding the antigen of interest could be fused to the DNA encoding the H22 heavy chain. The resulting fusion protein could enhance the processing and subsequent presention of the antigen to antigen-specific T cells by directing the antigen to CD64 found on the antigen-presenting cells [9, 16]. In this way, potent vaccines can be developed that can augment immunity to a given pathogen.

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