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DIMINUTION OF ANTIBODIES DIRECTED AGAINST TUMOR CELL SURFACE EPITOPES: A SINGLE CHAIN Fv FUSION MOLECULE SPECIFICALLY RECOGNIZES THE EXTRACELLULAR DOMAIN OF THE c-*erb*B-2 RECEPTOR

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Summary—We are evaluating strategies for the inhibition of growth or the selective killing of tumor cells. Cell surface antigens which are exclusively expressed or which are enhanced in their expression in tumor cells might provide the means to target cytotoxic or cytostatic agents to these cells. Few tumor specific cell surface antigens have been found, but the enhanced expression of growth factor receptors has been described for several types of tumors. A prominent example is the overexpression of the c-*erb*B-2 receptor in a high percentage of primary breast and ovarian carcinomas. We have derived monoclonal antibodies against the extracellular domain of the c-*erb*B-2 receptor. The antibody molecules were genetically engineered to minimize their size and to allow for their functional modification. For this purpose the cDNA sequences corresponding to the variable domains of one monoclonal antibody (FRP5) were molecularly cloned and joined by a short linker. The resulting single chain antibody molecule (scFv) was expressed in bacteria and purified. We show in an immunoprecipitation experiment that this molecule retains its ability to recognize the c-*erb*B-2 extracellular domain. This molecule could become a valuable vehicle to specifically transport anti-tumor agents to breast cancer cells.

INTRODUCTION

The use of serotherapy and antibodies for the treatment of cancer has occupied the scientific community for many years. The hope to exploit the specificity of the antigen–antibody interactions for this purpose has spurred the imagination [1–3]. Monoclonal antibodies (MAb) in tumor therapy have primarily been evaluated in hematologic malignancies [4], but melanomas and colon carcinomas have also been evaluated. Although some positive results have been obtained the overall success rate has been modest [5]. In the course of these investigations three major problem areas have been identified. First, there are limitations which are inherent to the MAbs and the distribution of the recognized antigens. They include the occurence of antigen in the circulation or other organs which is not associated with the tumor cell surface [6], the low and slow accrual of MAb in tumors and the immunogenicity of mouse antibody in humans. Second, there are limitations stemming from the nature of the antigens. Not many antigens fit the description of a "tumor specific surface antigen" and modulation and disappearance of the chosen antigen from the tumor cells has been observed. Third, the action of an unconjugated MAb is not necessarily cytotoxic, i.e. the response to a MAb can be expected to be transient, consistent with the short durations of the observed remissions.

Improvement of therapeutic applications in the future can be envisaged and methodological advances will ameliorate some of the limitations listed above [7]. Again, these advances can be grouped in three categories. First, advances are based on insights into the molecular biology of immunoglobulin (Ig) genes [8]. The molecular cloning of the Ig genes and progress in the manipulation and expression of cloned Ig genes has led to the development of single chain antibodies, composed merely of the variable light chain (VL) and the variable heavy chain (VH) sequence of the Ig molecule connected by a short linker. These single chain (scFv) constructs comprise about 25 kDa, as compared to the 150 kDa for an intact IgG molecule. The smaller size of the scFv constructs might be advantageous for the reduction of the human anti-mouse antibody (HAMA) response and for the increased accessibility of tumors *in vivo* [9].

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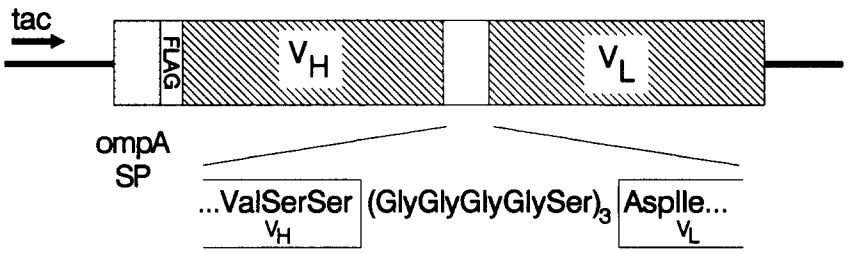

Fig. 1 Schematic representation of a bacterial expression vector for a single chain Fv fusion. The VH and VL domains of the MAb FRP5 (specific for the extracellular domain of the human c-*erb*B-2 receptor) were cloned by reverse transcription and PCR. The domains were joined by a linker sequence comprising three repeats of 5 amino acids The FLAG epitope, the ompA leader sequence were added to the 5′ end and the IPTG inducible tac promoter was used for the direction of transcription in transformed bacteria.

Second, MAb bound to drugs, toxins [10–12] or radioisotopes [13–15] might exhibit enhanced cytotoxic activity. In combination with the molecular manipulation techniques, it has become possible to design novel scFv–effector constructs. The smaller size of scFv might contribute to new coupling procedures to cytotoxic drugs and radioisotopes and allow the addition of toxin domains on the level of the cDNA. Genetic engineering might also allow a modification of their half life *in vivo*. Instability of the antibody-conjugates and side effects due to long half-lives might become avoidable. Third, the identification of oncogenes which are consistently involved in the etiology of specific tumors has progressed. Gene amplification and overexpression of the transmembrane receptor encoded by the c-*erb*B-2 proto-oncogene for example, has been observed in a high percentage of primary breast and ovarian carcinomas [16]. Although this receptor is not exclusively expressed in cancer cells, its expression in a subset of tumors is sufficiently enhanced over the expression in normal cells that it might be considered as a target for therapy.

The development of the components required for the targeted treatment of c-*erb*B-2 overexpressing cells include MAb specific for the extracellular domain of the receptor, the molecular cloning of scFv molecules, their expression in bacteria and the demonstration that these bacterially expressed molecules retain the ability to recognize the extracellular domain of the c-*erb*B-2 receptor. These steps are described here.

MATERIALS AND METHODS

Derivation of monoclonal antibodies

Female Balb/c mice were immunized four times with 2×10^7 SKBR3 cells over a period of 3 to 4 months. SKBR3 cells are human breast tumor cells which express approx. 1×10^6 molecules of c-*erb*B-2 receptor per cell. The mice were sacrificed and the spleen cells were fused with mouse myeloma cells, plated on peritoneal macrophages and selected in HAT medium. Hybridoma cells secreting a c-*erb*B-2 specific monoclonal antibody were identified in two steps. First, hybridoma supernatants were reacted with mouse cells transfected with the human c-*erb*B-2 proto-oncogene (HC11 mammary epithelial cell clone R1-11) and binding of antibodies was detected using fluorescin linked sheep anti-mouse IgG. Second, the positive supernatants were tested for their ability to immunoprecipitate the c-*erb*B-2 receptor of SKBR3 cells. The procedures have been described previously [17].

Molecular cloning and bacterial expression of a single chain Fv domain

FRP5 hybridoma cells express a c-*erb*B-2 specific MAb with a kappa VL and an IgG1 VH. mRNA was isolated from the hybridoma cell line and a reverse transcription reaction and a polymerase chain reaction (PCR) performed to clone the variable domains VH and VL of the Ig molecules. The primers for the reaction were chosen with the appropriate restriction sites at their ends so that the PCR products could be linked by a synthetic DNA sequence encoding 15 amino acids and cloned into a modified bluescript vector. This linker connects the VH and the VL regions in frame. The scFv was excised and recloned in the pFLAG-1 vector as shown in Fig. 1. This vector provides the scFv molecule with the FLAG epitope and subjects its bacterial expression to the IPTG inducible tac promoter. Bacteria were induced for 30 min with 0.5 mM IPTG and the scFv molecules were purified from periplasmic fractions or total

lysates by M1-MAb affinity column purification as suggested by IBI FLAG Biosystem manual [18]. The cloning of the scFv and expression of a scFv–phosphatase fusion protein have been described previously [19].

Coimmunoprecipitation of gp185, anti-gp185 and scFv

About 10^7 SKBR3 cells were lysed in 1 ml of Triton X-100 containing lysis buffer. 200 μl were incubated with 0.5 microgram of scFv and kept on ice for 1 h. 21N anti-serum, raised against a synthetic oligonucleotide which represents the C-terminal domain of the c-*erb*B-2 receptor, was added for 30 min, followed by protein A sepharose. The complex was dissolved in loading buffer, the proteins were separated on polyacrylamide gels and blotted onto nitrocellulose filters. 21N anti-serum was used to visualize the c-*erb*B-2 molecules and M1 anti-FLAG was used to visualize the scFv molecules in a Western procedure. Periplasmatic extracts from non-transformed *E. coli* cells (strain CC118) served as controls. The procedures have been described previously [19].

RESULTS AND DISCUSSION

The c-erbB-2 receptor is overexpressed in breast and ovarian carcinomas

The description of cancer as a disease which is mainly caused through the accumulation of mutations in somatic cells and the identification of oncogenes as mutated and activated counterparts of normal cellular proto-oncogenes has diminished the hope to find antigens which are consistently and exclusively present on the surface of tumor cells. Since immunotherapy is dependent on the occurence of cell surface antigens, the emphasis might shift from tumor specific antigens and their exclusive expression on the surface of tumor cells to antigens with enhanced expression on tumor cells. Such antigens might be oncogene products themselves or other cell surface proteins which become deregulated and overexpressed as a consequence of the activation of oncogenes.

The c-*erb*B-2 gene is a human proto-oncogene which encodes a growth factor receptor with a characteristic three domain structure. The receptor consists of an extracellular ligand binding domain, a single transmembrane domain and an intracellular domain with intrinsic tyrosine specific protein kinase activity. The c-*erb*B-2 receptor has extensive structure and sequence homology with the epidermal growth factor (EGF) receptor, but does not bind to EGF. A presumptive ligand for the c-*erb*B-2 receptor has recently been described [20]. The c-*erb*B-2 receptor has attracted the interest of many clinical investigations since it was found to be consistently involved in the development of breast and ovarian carcinomas [16]. The amplification of the c-*erb*B-2 gene and the overexpression of the c-*erb*B-2 receptor was found in a high percentage of human breast and ovarian tumor samples. These parameters have been correlated with the course of disease and c-*erb*B-2 expression has been established as a prognostic indicator. High c-*erb*B-2 expression predicts a shorter disease free and overall survival period of the cancer patients.

The c-erbB-2 receptor might constitute a target for therapy

The prognostic value of the determination of c-*erb*B-2 expression is not the only reason why this receptor is being intensively studied. The nature of this proto-oncogene makes it a prime candidate for experimental therapeutic approaches. At least four aspects of its genetic and biochemical properties might serve as a basis for this purpose. One, the receptor is a *bona fide* oncogene. The causal involvement of the receptor in cellular transformation *in vitro* has been shown in transfection experiments and the introduction of the receptor into transgenic mice has demonstrated its tumorigenic potential *in vivo*. These observations make it likely that interference with the activation of the receptor might result in the reversal of the transformed phenotype. Two, the receptor is dependent for its mitogenic signalling function, on the interaction with a specific ligand. The blockage of this interaction can be envisaged. MAb which prevent the interaction between the EGF receptor and EGF have been described and could serve as an example [21]. Three, at least some of the consequences of receptor activation are known. The interaction of the receptor with its ligand causes the activation of the intracellular tyrosine kinase domain. This tyrosine kinase phosphorylates cellular substrate proteins and sets off the mitogenic signalling cascade. It is conceivable that the enzymatic activity of the receptor or the activity of a crucial substrate could be specifically blocked by the action of drugs. Four, the receptor is a transmembrane protein with an extracellular domain which can

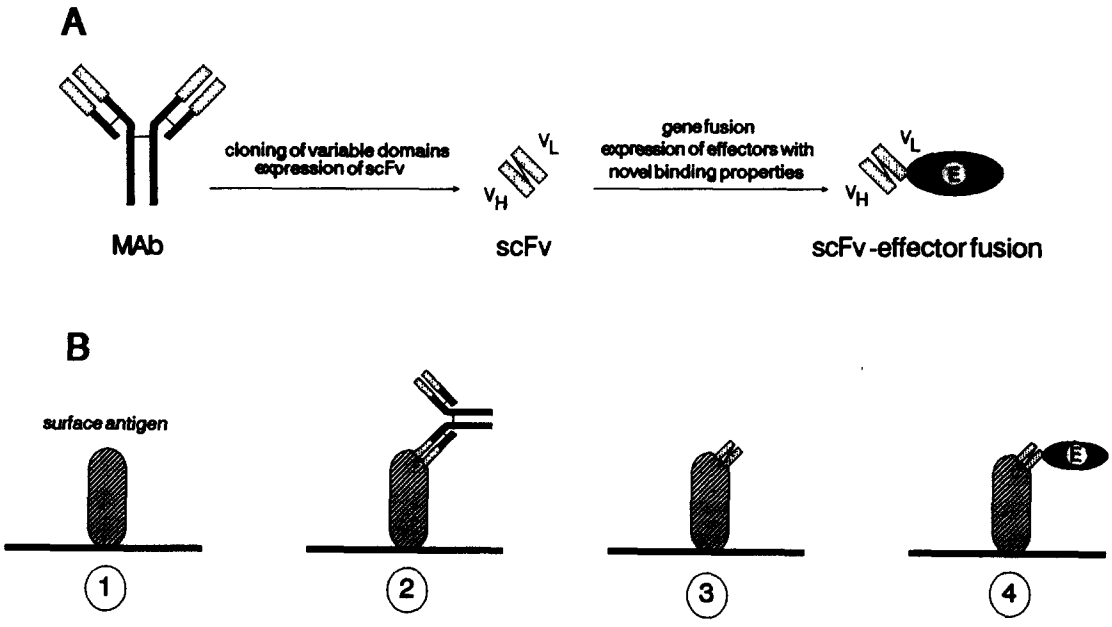

FIG 2 (A) The structure of a MAb is schematically shown The variable domains at the N-terminal ends of the heavy and light chains are indicated. These domains can be cloned as cDNA fragments and joined by a short linker sequence. The scFv molecule represents a single chain fusion of the VH and VL chain domains. The scFv molecules can be further modified by fusion with effector domains. These effector domains provide enzymatic properties and make the scFv–effector fusions bifunctional. The antigen recognition function is located in the scFv part of the molecule and the enzymatic function (e.g. toxin, prodrug activating function, radioisotope binding function) in the effector part. (B) Antigens on the surface of tumor cells can be used for targeting therapeutic agents. Monoclonal antibodies or scFv molecules can recognize such antigens. If the surface antigen is a growth factor receptor, the binding of the MAb or the scFv molecules might interfere with the binding of the activating ligand. The binding of scFv–effector fusions might potentiate the growth inhibiting effects

be recognized by antibodies directed against that domain.

Monoclonal antibodies against the c-erbB-2 receptor and single chain derivatives

The principle of antibody recognition of a cell surface antigen and the possibilities which are provided by progress in antibody engineering are illustrated in Fig. 2. MAb which specifically recognize the extracellular domain of c-*erb*B-2 have been obtained by using SKBR3 human breast carcinoma cells as an immunogen. These cells express a high number of c-*erb*B-2 receptor molecules on their surface. The use of intact cells in the immunization of mice makes it likely, that the antibodies obtained recognize the c-*erb*B-2 receptor in the *in vivo* setting. The MAb which we purified were selected for this criterion by a first screening step in which a cell binding assay was used [17].

MAb might on their own be useful for therapeutic purposes. As shown in Fig. 2 the MAb can bind the extracellular domain and could cause the blockage of receptor ligand interaction. This could be a mechanism to explain the effects of the MAb on the growth of transformed cells dependent on c-*erb*B-2 activation. Although such effects have been observed on SKBR3 cells [17], it is not possible at this point to attribute them to the ligand antagonism of the MAb. The availability of pure ligand will be a prerequisite to test the mechanism of growth inhibition. The experience with other MAb indicates that the action of murine MAb can be cytostatic and not necessarily cytotoxic.

Advances in the understanding of the structure of antibodies and in the methods of genetic engineering might help to change the situation. The antigen recognition domains of antibody molecules are well defined. They consist of the VH and the VL chain domains. Molecular cloning procedures have made it possible to obtain the respective cDNA sequences from the hybridoma cells. It is possible to join these sequences into a single oligonucleotide, providing them with a linker sequence encoding 15 amino acids. This artificial gene can be expressed in bacteria upon provision with a bacterial promoter sequence [22]. The construct shown in Fig. 1 is expressed in bacteria under

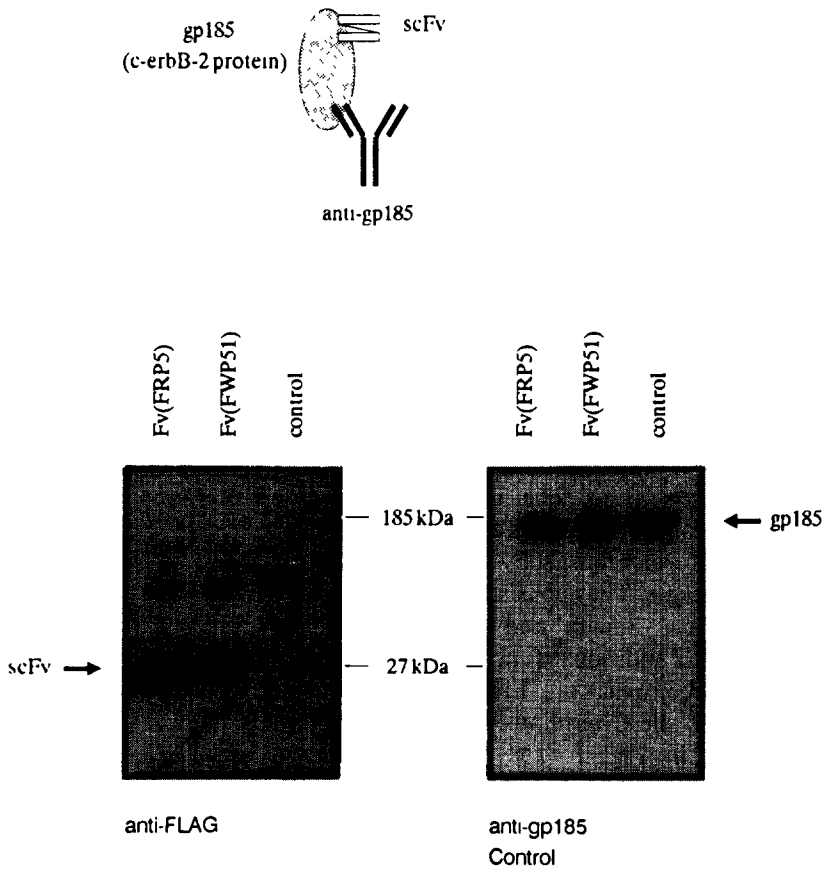

Fig. 3. The scFv molecules can recognize the c-*erb*B-2 receptor. Membrane extracts from SKBR3 cells were reacted with purified scFv molecules. 21N antibody, specific for a peptide on the C-terminus of the intracellular domain of the receptor was added and the complex of gp185 c-*erb*B-2, 21N antibody and scFv was purified by absorption to protein A sepharose. The complex was solubilized, electrophoresed and blotted and the blot was developed with anti-FLAG antibody or anti-gp185 c-*erb*B-2 antibody. scFv of 27 kDa derived from two MAb (FRP5 and FWP51) were recovered in the complex (left panel) and could be visualized with the anti-FLAG antibody. The presence of gp185 c-*erb*B-2 was visualized with the 21N antibody (right panel). When periplasmic extract from non-transformed bacteria was used (control) only the gp185 c-*erb*B-2 was present in the complex.

the control of the IPTG inducible tac promoter. In addition to the components mentioned, the construct shown in Fig. 1 has been provided with the FLAG epitope and the ompP sequence. Both sequences are included to facilitate the recovery of the protein from the bacterial cultures. The FLAG epitope allows the purification of the bacterially expressed gene by affinity purification with the MAb M1 and the ompA sequence causes the secretion of the protein into the periplasmic space. Figure 2 shows the derivation of the scFv construct and its binding to the cell surface antigen. In contrast to the MAb which is bivalent with respect to antigen recognition, the scFv molecule has only a single antigen recognition site.

After the extensive engineering of the antigen recognition domain it is necessary to demonstrate that the bacterially expressed protein retains its ability to recognize its antigen. Figure 3 shows an experiment in which the complex formation between the scFv molecule and the gp185 c-*erb*B-2 protein was tested. Purified scFv was added to a membrane extract of SKBR3 cells. The scFv molecule formed a complex with the extracellular domain of gp185 c-*erb*B-2. This complex was reacted with a second gp185 c-*erb*B-2 specific antibody, 21N, which recognizes a peptide on the C-terminus of the intracellular domain. The scFv recognizing the extracellular domain and the 21N antibody recognizing the intracellular domain of c-*erb*B-2 are not expected to interfere with each other. The complex of all three molecules was bound to protein A sepharose via the 21N antibody, solubilized in SDS buffer, electrophoresed and blotted onto a nitrocellulose membrane. The presence of scFv in the complex was visualized

by developing the blot with an anti-FLAG MAb, M1 and the presence of the gp185 c-*erb* B-2 by developing the blot with the 21N antibody. This experiment shows that scFv has retained its ability to bind to gp185 c-*erb* B-2.

Bifunctional derivatives of scFv might become useful in the detection and elimination of tumor cells

The effects which scFv molecules have on the growth properties of cells have not yet been well studied. If a scFv is able to interfere with ligand binding, one would predict that its effect should be similar to that observed with intact MAb. If the effect of the MAb is based on receptor crosslinking and downregulation, one might expect the scFv effect to be less pronounced than that of the MAb. Nevertheless, scFv molecules might offer advantages. They consist of a single antigen recognition domain and they can be supplemented with additional functions by means of *in vitro* recombination. Effector domains can be added to the C-terminus. This is schematically shown in Fig. 1. The scFv domain could serve as a vector for the delivery of potentially cytotoxic functions to the surface of tumor cells. Three classes of effector domains have been considered. First, protein toxins such as exotoxin A from Pseudomonas, diphtheria toxin and ricin could become useful. These toxins inhibit cellular protein synthesis upon their uptake into cells and kill them very efficiently. They have been coupled by chemical means to various antibodies to create so called "immunotoxins". The coupling procedures and the non-specific toxicity of these conjugates were not always satisfactory. The recent gene constructs in which growth factors and cytokines or single chain antibodies were fused with the enzymatic domains of toxins seem more promising [23–25]. Second, radioisotopes of medium or high beta energy like 131 iodine or 90 yttrium have been coupled to MAb and used in clinical trials. The emission of electrons causes damage to several cell layers in the vicinity of the decay event. As in the case of antibody–toxin coupling, the methods to attach the radioisotopes to the large antibody molecules are not perfected. Genetically engineered, smaller scFv molecules might offer advantages for the association with radioisotopes [13, 14]. Third, antibodies have been used to deliver enzymes capable of activating "prodrugs" to the surface of tumor cells. The prodrugs can be converted into cytotoxic agents by the release of a modifying group. The release of etoposide from etoposide–phosphate or mitomycin from mitomycin–phosphate might serve as examples [26–28]. The efficiency with which cytotoxic activity can be generated at the site of cancer cells through the activation of prodrugs may also be improved through the *in vitro* recombination of bifunctional scFv molecules.

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