SUMMARY ~~OF THE INVENTION~~

The present ~~invention~~disclosure provides novel anti-tumor agents and methods for the treatment of cancer.

The present ~~invention~~disclosure is based on the surprising finding that GPI-anchored TIMP effectively reduces or alleviates cancer cell ~~proliferation,~~ and promotes the killing of cancer cells in cell lines both in vitro and in vivo. The structural and functional determinants of TIMP have been combined with a glycosylphosphatidylinositol (GPI) anchor, and, optionally, with mucin, in order to generate a highly effective chemotherapy agent. This approach exploits TIMP proteins anchored by glycosylphosphatidylinositol (GPI) to be incorporated into surface membranes when purified and added to cancer cells. The fusion of TIMP-GPI with a mucin domain further enhances the presentation of TIMP proteins on the surface cell membrane and makes the fusion construct more effective in rendering the cancer cells sensitive to immune-mediated destruction.

In the following examples, the present ~~invention~~disclosure demonstrates that TIMP has the potential for the inhibition of growth of tumor cells and reduction of tumor development both in cell lines in vitro and in tissues in vivo. The linking of TIMP to a GPI-anchor and exogenous administration of GPI-anchored TIMP results in an efficient insertion of TIMP protein into the cell membranes of cancer cells. The GPI-anchored TIMP-1 surface expression induced a variety of biological effects in cancer cell lines with potential therapeutic relevance such as inducing the FAS-mediated apoptotic pathway in cancer cells. As shown in the following examples, the suppression of cancer cell proliferation was observed to be dose-dependent.

GPI-anchored TIMP-1 protein also blocked secretion of proMMP-2 and proMMP-9 and dramatically altered the cell surface association of diverse MMPs. Most significantly, the normally FAS-apoptosis resistant tumor cell lines were rendered sensitive to FAS/CD95-mediated killing. GPI-TIMP treatment results in a down regulation of anti-apoptotic BCL2 protein and a corresponding increase in pro-apoptotic BAX protein. This shift towards a higher concentration of pro-apoptotic proteins may be one reason for the increased sensitivity of FAS-mediated apoptosis of TIMP surface engineered cancer cells.

Using the above approach, the GPI-anchored TIMP proteins or polypeptides of the present ~~invention~~disclosure have been proved to be particularly useful in therapeutic applications in the treatment of residual cancer after incomplete surgical resection of the primary tumor like in advanced breast cancer, osteosarcoma, renal cell carcinoma or in malignant brain tumors, e.g. glioblastoma.

Moreover, since tumor cells, including renal cell carcinoma (RCC), are intrinsically resistant to FAS- mediated killing, the present ~~invention~~disclosure provides an effective means to render the tumor cells susceptible to FAS-mediated apoptosis.

In a first ~~aspect~~embodiment, the present ~~invention~~disclosure therefore relates to a fusion construct (TIMP-GPI or TIMP-mucin-GPI) comprising an amino acid sequence of a tissue inhibitor of metalloproteinases (TIMP) or a biologically active fragment thereof, wherein said TIMP or biologically active fragment thereof is linked to an amino acid sequence of a mucin domain followed by an amino acid sequence of a glycosylphosphatidylinositol (GPI)-anchor.

In a preferred embodiment, the 3´-end of TIMP is fused directly to a GPI-linking sequence and does not contain a mucin domain.

The term "mucin" relates to a family of large, heavily glycosylated proteins. One class of mucins are membrane-bound due to the presence of a hydrophobic membrane-spanning domain that favors retention in the plasma membrane, while another class of mucins are secreted on mucosal surfaces. Mucin genes encode mucin monomers that are typically synthesized as rod-shape apomucin cores that are post-translationally modified by exceptionally abundant glycosylation. Two distinctly different regions are found in mature mucins. One region includes the amino- and carboxy-terminal regions, which are lightly glycosylated, but rich in cysteine residues, which are likely involved in establishing disulfide linkages within and among mucin monomers. The second central region is formed of multiple tandem repeats of 10 to 80 residue sequences, wherein over half of the amino acid residues are serine or threonine.

Mucins are generally secreted as massive aggregates of proteins having molecular masses of roughly 1 to 10 million Daltons. Within these aggregates, monomers are linked to one another mostly by non-covalent interactions, although intermolecular disulfide bonds may also play a

role in this process. At least 19 human mucin genes have been distinguished including MUC1, 2, 3A, 3B, 4, 5AC, 5B, 6-9, 11-13, and 15-19.

The mucin as used in the present ~~invention~~disclosure is preferably a membrane-bound mucin domain and comprises preferably an amino acid sequence selected from the group consisting of MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC16, and MUC17, or a variant or portion thereof (the above mucins are reviewed in Moniaux N *et al.*, 2004). In another preferred embodiment, a mucin-stalk is used that is isolated from the surface-associated chemokine CXCL16 or fractalkine (CX3CL1).

Fractalkine is a member of the large and complex chemokine gene superfamily, which consists principally of secreted, proinflammatory molecules. The typical core structure of chemokines is partially maintained by disulfide bonds between positionally conserved cysteine resides. For most of chemokine peptides, a familiar structural characteristic is the distribution of four cysteines within the molecule, i.e. a cysteine signature motif: CXC, CC and C, where C is a cysteine and X is any amino-acid residue. Four different chemokine families have been identified based on the observation that chemokine peptides can be distinguished by the organization of the cysteine residues located near the N-terminus of the molecule. Fractalkine itself defines one of the chemokine families, and is distinguished structurally from other chemokine families as the N-terminal cysteines of fractalkine are separated by three residues (i.e. a CX3C motif) as well as being tethered to the cell membrane by an extended C-terminus transmembrane anchor that includes a mucin-like domain, or a mucin-like stalk. Thus, the mucin and fractalkine domains contained within the fusion constructs of the present ~~invention~~disclosure are suitable for achieving an improved anchoring of the TIMP protein in the cell membrane.

The TIMP as used in the present disclosure is preferably derived from a mammal; more preferred is a human (the four TIMPs are reviewed in Mannello F *et al.*, 2001). Examples of TIMP proteins that can be used in accordance with the present ~~invention~~disclosure comprise TIMP-1, TIMP-2, TIMP-3, or TIMP-4 and their corresponding variants in other organisms such as mouse, rabbit, dog, cat, sheep, and cow.

The GPI-anchor as used in the present ~~invention~~disclosure is preferably derived from the lymphocyte function-associated antigen (LFA-3), or a portion thereof, and includes a GPI-signal sequence that mediates membrane association.

The present ~~invention~~disclosure also relates to a nucleic acid molecule, such as RNA or DNA, comprising a nucleic acid sequence that encodes for the GPI-anchored TIMP construct of the ~~invention~~disclosure.

In a further ~~aspect~~embodiment of the present ~~invention~~disclosure, the nucleic acid molecule of the ~~invention~~disclosure is contained in an expression plasmid, a vector or a host cell for expression of the nucleic acid molecule of the ~~invention~~disclosure.

The present ~~invention~~disclosure also relates to the use of the TIMP-GPI- or TIMP-mucin-GPI fusion constructs of the ~~invention~~disclosure for the treatment of cancer, particularly residual cancer after surgical removal of a primary tumor.

In one aspect, there is provided the use of a fusion construct comprising an amino acid sequence of a tissue inhibitor of metalloproteinases (TIMP) or a biologically active fragment thereof, that inhibits matrix active metalloproteinases, wherein said TIMP or biologically active fragment thereof is linked to a glycosylphosphatidylinositol (GPI)-anchor for the preparation of a medicament for the treatment of cancer.

In a further ~~aspect~~embodiment of the present ~~invention~~disclosure, the TIMP-GPI or TIMP-mucin-GPI fusion constructs of the ~~invention~~disclosure are contained in a pharmaceutical composition or medicament. In a further embodiment, the TIMP-GPI or TIMP-mucin-GPI fusion constructs of the ~~invention~~disclosure are suitably used as anti-cancer agents or drugs. In a preferred embodiment, the anti-cancer drugs of the ~~invention~~disclosure are administered and applied locally to the side of the tumor mass resection in high-risk tumor patients with a high risk of residual cancer cells and increased incidence of a local relapse, and in those patients having an obvious residual tumor due to advanced stage disease or local inoperability. Preferably, the fusion construct is administered by spraying into the wound and/or injection into regions that are not available for surgery.

In one aspect, there is provided an *in-vitro* method for inhibition of cancer cell proliferation comprising the steps of:

(1) subjecting a cancer cell line to an effective amount of TIMP-GPI fusion construct.

The present ~~invention~~disclosure also relates to an *in-vitro* method for inhibition of cancer cell proliferation comprising the steps of subjecting a cancer cell line to an effective amount of TIMP-mucin-GPI or TIMP-GPI fusion construct.

In one aspect, there is provided the use of TIMP-GPI for rendering FAS-apoptosis resistant tumour cell lines sensitive to FAS-induced apoptosis.

In a further embodiment, the present ~~invention~~disclosure provides novel agents and methods for the treatment of conditions defined by a disturbed balance between normal physiological MMP protease activity and ECM deposition, which result in abnormal wound healing. In one embodiment, the present ~~invention~~disclosure provides agents and methods suitable for the treatment of

keloid or hypertrophic scarring and chronic wounds commonly associated with increased MMP levels. Furthermore, the present ~~invention~~disclosure also provides effective agents and methods for reducing, minimizing or inhibiting the formation of scars during the wound healing process.

DEFINITIONS

With the term "TIMP" as used herein is meant an endogenous tissue inhibitor of metalloproteinases, which is known to be involved in physiological/biological functions including the inhibition of active matrix metalloproteinases, regulation of pro MMP activation, cell growth, and the modulation of angiogenesis. The human "TIMP family" contains four members, TIMP-1, TIMP-2, TIMP-3, and TIMP-4. One preferred member used in the present invention, TIMP-1, is a secreted protein that can be detected on the cell surface through its interaction with surface proteins (Bode & Maskos, 2003).

The term "fusion construct" or "TIMP fusion construct" as used herein refer to both the nucleic acid molecule and the amino acid molecule encoded thereby.

The invention specifically relates to nucleic acids containing a nucleotide sequence including the sequence defined by SEQ ID NOS:1-5, or a homolog thereof, or unique fragments thereof. In the present invention, the sequence of a nucleic acid molecule that encodes the resulting protein is considered homologous to a second nucleic acid molecule if the nucleotide sequence of the first nucleic acid molecule is at least about 70% homologous, preferably at least about 80% homologous, and more preferably at least about 90% homologous to the sequence of the second nucleic acid molecule. Homology between two nucleic acid sequences may be readily determined using the known BLASTN algorithm (Altschul, *et al.*, 1990) with default settings. As a further example, another known test for ascertaining the homology of two nucleic acid sequences is whether they hybridize under normal hybridization conditions, preferably under stringent hybridization ~~conditions~~.

Given the nucleic acid sequence disclosed herein, the skilled person can readily design nucleic acid structures having particular functions in various types of applications. For example, the artisan can construct oligonucleotides or polynucleotides for use as primers in nucleic acid amplification procedures, such as the polymerase chain reaction (PCR), ligase