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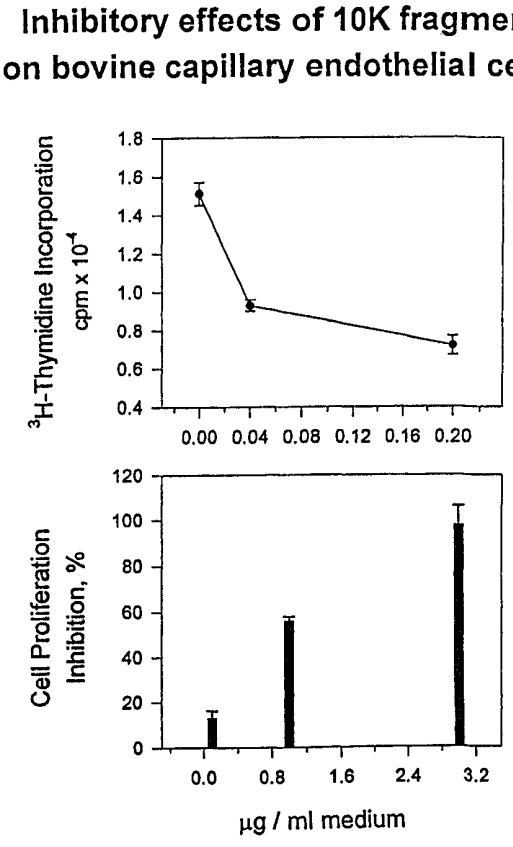
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(54) Title: PLASMINOGEN KRINGLE 4 REGION FRAGMENTS AND METHODS OF USE

(57) Abstract

Fragments of an endothelial cell proliferation inhibitor and method of use therefor are provided. The endothelial proliferation inhibitor is a protein derived from plasminogen, or more specifically is an angiostatin kringle 4 region fragment. The kringle 4 region fragments generally correspond to kringle 4 structures occurring within the endothelial cell proliferation inhibitor. The endothelial cell inhibiting activity of these fragments provides a means for inhibiting angiogenesis of tumors and for treating angiogenic–mediated disease.



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PLASMINOGEN KRINGLE 4 REGION FRAGMENTS AND METHODS OF USE

Related Applications

The present application claims priority to U.S. Provisional Application Serial No. 60/117,617 filed January 28, 1999.

Field of the Invention

The present invention relates to endothelial inhibitors, fragments of angiostatin protein, which reversibly inhibit proliferation of endothelial cells. More particularly, the present invention relates to kringle 4 region fragments that are useful for the treatment of angiogenesis-associated diseases such as cancer.

Background of the Invention

As used herein, the term "angiogenesis" means the generation of new blood vessels into a tissue or organ. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonal development and formation of the corpus luteum, endometrium and placenta. The term "endothelium" means a thin layer of flat epithelial cells that lines serous cavities, lymph vessels, and blood vessels.

Both controlled and uncontrolled angiogenesis are thought to proceed in a similar manner. Endothelial cells and pericytes,

surrounded by a basement membrane, form capillary blood vessels. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic stimulants induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" off the parent blood vessel, where the endothelial cells undergo mitosis and proliferate. The endothelial sprouts merge with each other to form capillary loops, creating the new blood vessel.

Persistent, unregulated angiogenesis occurs in a multiplicity of disease states, tumor metastasis and abnormal growth by endothelial cells. The diverse pathological disease states in which unregulated angiogenesis is present have been grouped together as angiogenic dependent or angiogenic associated diseases.

The hypothesis that tumor growth is angiogenesis-dependent was first proposed in 1971. (Folkman J., Tumor angiogenesis: Therapeutic implications., *N. Engl. Jour. Med.* 285:1182 1186, 1971) In its simplest terms it states: "Once tumor 'take' has occurred, every increase in tumor cell population must be preceded by an increase in new capillaries converging on the tumor." Tumor 'take' is currently understood to indicate a prevascular phase of tumor growth in which a population of tumor cells occupying a few cubic millimeters volume and not exceeding a few million cells, can survive on existing host microvessels. Expansion of tumor volume beyond this phase requires the induction of new capillary blood vessels.

Amino acid sequence alignment of the kringle domains of human plasminogen, designated K1, K2, K3 and K4, shows that all kringle regions display identical gross architecture and remarkable sequence homology (56-82% identify). Among these structures, the high-affinity lysine binding kringle, K1, is

the most potent inhibitory segment of endothelial cell proliferation. Of interest, the intermediate-affinity lysine binding fragment, K4, has previously been shown to lack inhibitory activity. These data suggest that the lysine binding site of the kringle structures may not be directly involved in the inhibitory activity. The amino acid conservation and functional divergence of these kringle structures provide an ideal system to study the role mutations caused by DNA replication during evolution. Similar divergent activities relative to the regulation of angiogenesis exhibited by a group of structurally related proteins are also found in the -C-X-C- chemokine and prolactin-growth hormone families (Maione, T.E., Gray, G.S., Petro, A. J., Hunt, A.L., and Donner, S.I. (1990) *Science* 247, 77-79.; Koch, A.E., Polverini, P.J., Kunkel, S.L., Harlow, L.A., DiPietro, L.A., Elner, V.M., Elner, S.J., and Strieter, R.M. (1992) *Science* 258, 1798-1801.; Cao, Y., Chen, C., Weatherbee, J.A., Tsang, M., and Folkman, J. (1995) *J. Exp. Med.* 182, 2069-2077.; Strieter, R.M., Polverini, P.J., Arenberg, D.A., and Kunkel, S.L. (1995) *Shock* 4, 155-160.; Jackson, D., Volpert, O.V., Bouck, N., and Linzer, D.I.H. (1994) *Science* 266, 1581-1584).

Further sequence analysis reveals that K4 contains two positively charged lysine residues adjacent to cysteines 22 and 78 (Fig. 35). ^{1}H nuclear magnetic resonance (NMR) analysis shows that these 4 lysines, together with lysine 57, form the core of a positively charged domain in K4 (Llinas M, unpublished data), whereas other kringle structures lack such a positively charged domain. Whether this lysine-enriched domain contributes to the loss of inhibitory activity of kringle 4 of human plasminogen remains to be studied. K4 was previously reported to stimulate proliferation of other cell types and to increase the release of intracellular calcium (Donate, L.E., Gherardi, E., Srinivasan, N., Sowdhamini, R., Aporicio, S., and Blundell, T. L. (1994) *Prot. Sci.* 3, 2378-2394). The fact that removal of K4 from angiostatin potentiates its inhibitory activity on endothelial cells

suggests that this structure may prevent some of the inhibitory effect of K1-3.

The mechanism underlying how angiostatin and its related kringle fragments specifically inhibit endothelial cell growth remains uncharacterized. It is not yet clear whether the inhibition is mediated by a receptor that is specifically expressed in proliferating endothelial cells, or if angiostatin is internalized by endothelial cells and subsequently inhibits cell proliferation. Alternatively, angiostatin may interact with an endothelial cell adhesion receptor such as integrin a_vb_3, blocking integrin-mediated angiogenesis (Brooks, P.C., Montgomery, A.M., Rosenfeld, M., Reisfeld R.A., Hu, T. Klier, G., and Cheresh, D.A. (1994) *Cell* 79, 1157-1164). Of interest, Friedlander et. al. (Friedlander, M., Brooks, P.C., Shaffer, R.W., Kincaid, C.M., Varner, J.A., and Cheresh, D.A. (1995) 270, 1502) reported recently that *in vivo* angiogenesis in cornea or chorioallantoic membrane models (induced by bFGF and by tumor necrosis factor) was a_vb_3 integrin dependent. However, angiogenesis stimulated by VEGF, transforming growth factor a, or phorbol esters was dependent on a_vb_5. Antibodies to the individual integrins specifically blocked one of these pathways, and a cyclic protein antagonist of both integrins blocked angiogenesis induced by each cytokine (Friedlander, M., Brooks, P.C., Shaffer, R.W., Kincaid, C.M., Varner, J.A., and Cheresh, D.A. (1995) 270, 1502). Because both bFGF- and VEGF-induced angiogenesis are inhibited by angiostatin, angiostatin may block a common pathway involved in integrin-mediated angiogenesis.

An increasing number of endogenous angiogenesis inhibitors have been identified in the last few decades (Folkman, J. (1995) *N. Engl. J. Med.* 333, 1757-1763). Of the nine characterized endothelial cell suppressors, several inhibitors are proteolytic fragments. For example, the 16 kDa N-terminal fragment of human prolactin inhibits endothelial cell proliferation and blocks angiogenesis *in vivo* (Clapp, C., Martial, J.A., Guzman, R.C., Rentierdelrue, F., and Weiner, R.I. (1993)

Endorinology 133, 1292-1299). In a recent paper, D'Angelo et. al. reported that the antiangiogenic 16 kDa N-terminal fragment inhibited the activation of mitogen-activated protein kinase (MAPK) by VEGF and bFGF in capillary endothelial cells (D'Angelo, G., Struman, I., Martial, J., and Weiner, R. (1995) *Proc. Natl. Acad. Sci.* 92, 6374-6378). Similar to angiostatin, the intact parental molecule of prolactin does not inhibit endothelial cell proliferation nor is it an angiogenesis inhibitor. Platelet factor 4 (PF-4) inhibits angiogenesis at high concentrations (Maione, T.E., Gray, G.S., Petro, A. J., Hunt, A.L., and Donner, S.I. (1990) *Science* 247, 77-79; Cao, Y., Chen, C., Weatherbee, J.A., Tsang, M., and Folkman, J. (1995) *J. Exp. Med.* 182, 2069-2077). However, the N-terminally truncated proteolytically cleaved PF-4 fragment exhibits a 30- to 50-fold increase in its anti-proliferative activity over the intact PF-4 molecule (Gupta, S.K., Hassel, T., and Singh, J.P. (1995) *Proc. Natl. Acad. Sci.* 92, 7799-7803). Smaller protein fragments of fibronectin, murine epidermal growth factor, and thrombospondin have also been shown to specifically inhibit endothelial cell growth (Homandberg, G.A., Williams, J.E., Grant, D., Schumacher, B., and Eisenstein, R. (1985) *Am. J. Pathol.* 120, 327-332; Nelson, J., Allen, W.E., Scott, W.N., Bailie, J.R., Walker, B., McFerran, N.V., and Wilson, D.J. (1995) *Cancer Res.* 55, 3772-3776; Tolsma, S.S., Volpert, O.V., Good, D.J., Frazer, W.A., Polverini, P.J., and Bouck, N. (1993) *J. Cell Biol.* 122, 497-511). Proteolytic processing of a large protein may change the conformational structure of the original molecule or expose new epitopes that are antiangiogenic. Thus, protease(s) may play a critical role in the regulation of angiogenesis. To date, little is known about the regulation of these protease activities *in vivo*.

The data also show that the disulfide bond mediated folding of the kringle structures in angiostatin is preferable to maintain its inhibitory activity on endothelial cell growth. Kringle structures analogous to those in plasminogen are also

found in a variety of other proteins. For example, apolipoprotein (a) has as many as 37 repeats of plasminogen kringle 4 (McLean, J.W., Tomlinson, J.E., Kuang, W.-J., Eaton, D.L., Chen, E.Y., Fless, G.M., Scanu, A.M., and Lawn, R.M. (1987) *Nature* 330, 132-137). The amino terminal portion of prothrombin also contains two kringles that are homologous to those of plasminogen (Walz, D.A., Hewett-Emmett, D., and Seegers, W.H. (1977) *Proc. Natl. Acad. Sci.* 74, 1969-1973). Urokinase has been shown to possess a kringle structure that shares extensive homology with plasminogen (Gunzler, W.A., J., S.G., Otting, F., Kim, S.-M. A., Frankus, E., and Flohe, L. (1982) *Hoppe-Seyler's A. Physiol. Chem.* 363, 1155-1165). In addition, surfactant protein B and hepatocyte growth factor (HGF), also carry kringle structures (Johansson, J., Curstedt, T., and Jörnvall., H. (1991) *Biochem.* 30, 6917-6921; Lukker, N.A., Presta, L.G., and Godowski, P.J. (1994) *Prot. Engin.* 7, 895-903).

Thus, it is clear that angiogenesis plays a major role in the metastasis of a cancer. If this angiogenic activity could be repressed or eliminated, then the tumor, although present, would not grow. In the disease state, prevention of angiogenesis could avert the damage caused by the invasion of the new microvascular system. Therapies directed at control of the angiogenic processes could lead to the abrogation or mitigation of these diseases.

What is needed therefore is a composition and method which can inhibit the unwanted growth of blood vessels, especially into tumors. Also needed is a method for detecting, measuring, and localizing the composition. The composition should be able to overcome the activity of endogenous growth factors in pre-metastatic tumors and prevent the formation of the capillaries in the tumors thereby inhibiting the growth of the tumors. The composition, fragments of the composition, and antibodies specific to the composition, should also be able to modulate the formation of capillaries in other angiogenic

processes, such as wound healing and reproduction. The composition and method for inhibiting angiogenesis should preferably be non-toxic and produce few side effects. Also needed is a method for detecting, measuring, and localizing the binding sites for the composition as well as sites of biosynthesis of the composition. The composition and fragments of the composition should be capable of being conjugated to other molecules for both radioactive and non-radioactive labeling purposes

Summary of the Invention

In accordance with the present invention, compositions and methods are provided that are effective for modulating angiogenesis, and inhibiting unwanted angiogenesis, especially angiogenesis related to tumor growth. The present invention relates to a protein, which has been named "angiostatin", defined by its ability to overcome the angiogenic activity of endogenous growth factors such as bFGF, *in vitro*, and by its amino acid sequence homology and structural similarity to an internal portion of plasminogen beginning at approximately amino acid 98. Angiostatin comprises a protein having a molecular weight of between approximately 38 kilodaltons and 45 kilodaltons as determined by reducing polyacrylamide gel electrophoresis and having an amino acid sequence substantially similar to that of a fragment of murine plasminogen beginning at amino acid number 98 of an intact murine plasminogen molecule. Angiostatin protein contains approximately kringle regions 1 through 4 of a plasminogen molecule.

The present invention relates to fragments of angiostatin protein in the kringle 4 region. The amino acid sequences of the kringle 4 region fragments of the present invention vary slightly depending upon the species. Furthermore, the amino acid sequences of the kringle 4 region fragments of the present invention vary slightly at the amino and carboxy terminals. Therefore, it is to be understood that the number of amino acids

in the active kringle 4 region fragments may vary and all kringle 4 region amino acid sequences that have endothelial inhibiting activity are contemplated as being included in the present invention. The present invention also includes fusion proteins containing kringle 4 region fragments and other anti-angiogenic or angiogenic molecules. Examples of other anti-angiogenic molecules include endostatin protein and fragments of endostatin protein.

The present invention provides methods and compositions for treating diseases and processes mediated by undesired and uncontrolled angiogenesis by increasing the *in vivo* concentrations of kringle 4 region fragments in a human or animal. The *in vivo* concentrations of kringle 4 region fragments may be increased by administering to a human or animal a composition comprising a substantially purified kringle 4 region fragment in a dosage sufficient to inhibit angiogenesis. Additionally, the *in vivo* concentrations of kringle 4 region fragments may be increased in a human or animal by the administration of nucleotides encoding kringle 4 region fragments or enzymes that release kringle 4 region fragments from plasminogen or angiostatin. The present invention is particularly useful for treating, or for repressing the growth of, tumors. Increasing the *in vivo* concentrations of kringle 4 region fragments in a human or animal with prevascularized metastasized tumors will prevent the growth or expansion of those tumors.

The present invention also encompasses DNA sequences encoding kringle 4 region fragments or kringle 4 region fusion proteins, expression vectors containing DNA sequences encoding kringle 4 region fragments or kringle 4 region fusion proteins, and cells containing one or more expression vectors containing DNA sequences encoding kringle 4 region fragments or kringle 4 region fusion proteins. The present invention further encompasses gene therapy methods whereby DNA sequences encoding kringle 4 region fragments are introduced

into a patient to modify *in vivo* angiostatin kringle 4 region levels.

The present invention also includes diagnostic methods and kits for detection and measurement of kringle 4 region fragments in biological fluids and tissues, and for localization of kringle 4 region fragments in tissues and cells. The diagnostic method and kit can be in any configuration well known to those of ordinary skill in the art. The present invention also includes antibodies specific for the kringle 4 region fragments and portions thereof, and antibodies that inhibit the binding of antibodies specific for the kringle 4 region fragments. These antibodies can be polyclonal antibodies or monoclonal antibodies. The antibodies specific for the kringle 4 region fragments can be used in diagnostic kits to detect the presence and quantity of angiostatin which is diagnostic or prognostic for the occurrence or recurrence of cancer or other diseases mediated by angiogenesis. Antibodies specific for kringle 4 region fragments may also be administered to a human or animal to passively immunize the human or animal against angiostatin, or kringle 4 region fragments of angiostatin, thereby reducing angiogenic inhibition.

The present invention also includes diagnostic methods and kits for detecting the presence and quantity of antibodies that bind kringle 4 region fragments in body fluids. The diagnostic method and kit can be in any configuration well known to those of ordinary skill in the art. The present invention also includes antibodies that specifically bind to the angiostatin kringle 4 region receptor and transmit the appropriate signal to the cell and act as agonists or antagonists.

The present invention also includes kringle 4 region fragments and analogs that can be labeled isotopically or with other molecules or proteins for use in the detection and visualization of angiostatin fragment binding sites with techniques, including, but not limited to, positron emission

tomography, autoradiography, flow cytometry, radioreceptor binding assays, and immunohistochemistry.

The kringle 4 region fragments and analogs of the present invention also act as agonists and antagonists at the angiostatin kringle 4 region receptor, thereby enhancing or blocking the biological activity of angiostatin kringle 4 regions. Such proteins are used in the isolation of kringle 4 region fragments receptors.

The present invention also includes kringle 4 region fragment antisera, or angiostatin kringle 4 region receptor agonists and receptor antagonists linked to cytotoxic agents for therapeutic and research applications. Still further, kringle 4 region fragments, kringle 4 region fragment antisera, kringle 4 region fragment receptor agonists and kringle 4 region fragment receptor antagonists are combined with pharmaceutically acceptable excipients, and optionally sustained-release compounds or compositions, such as biodegradable polymers, to form therapeutic compositions.

The present invention includes molecular probes for the ribonucleic acid and deoxyribonucleic acid involved in transcription and translation of kringle 4 region fragments. These molecular probes provide means to detect and measure angiostatin kringle 4 region biosynthesis in tissues and cells.

Accordingly, it is an object of the present invention to provide a composition comprising a kringle 4 region.

It is another object of the present invention to provide a method of treating diseases and processes that are mediated by angiogenesis.

It is another object of the present invention to provide compositions and methods for increasing the *in vivo* concentration of kringle 4 region peptides.

It is an object of the present invention to provide compounds that modulate or mimic the production or activity of enzymes that produce kringle 4 region fragments *in vivo* or *in vitro*.

It is yet another object of the present invention to provide a diagnostic or prognostic method and kit for detecting the presence and amount of a kringle 4 region peptide in a body fluid or tissue.

It is another object of the present invention to provide a composition for treating or repressing the growth of a cancer.

It is a further object of the present invention to provide kringle 4 region or anti-kringle 4 region peptide antibodies by direct injection of angiostatin kringle 4 region DNA into a human or animal needing such kringle 4 region or anti-kringle 4 region peptide antibodies.

It is an object of present invention to provide a method for detecting and quantifying the presence of an antibody specific for a kringle 4 region fragment in a body fluid.

Still another object of the present invention is to provide a composition consisting of antibodies to kringle 4 region fragments that are selective for specific regions of the kringle 4 region fragment molecule that do not recognize plasminogen.

It is another object of the present invention to provide a method for the detection or prognosis of cancer.

It is another object of the present invention to provide a composition for use in visualizing and quantitating sites of kringle 4 region fragment binding *in vivo* and *in vitro*.

It is yet another object of the present invention to provide a therapy for cancer that has minimal side effects.

Still another object of the present invention is to provide a composition comprising kringle 4 region fragments linked to a cytotoxic agent for treating or repressing the growth of a cancer.

Another object of the present invention is to provide a method for targeted delivery of kringle 4 region-related compositions to specific locations.

Yet another object of the invention is to provide compositions and methods useful for gene therapy for the modulation of angiogenic processes.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

Brief Description of the Figures

Fig. 1 shows the production of recombinant murine angiostatin with a baculovirus expression system.

Fig. 2 shows a gel filtration chromatography of angiostatin degradation products.

Fig. 3 shows production of fragments from a 52 kDa recombinant murine angiostatin.

Fig. 4 shows the inhibitory effects of a 10 kDa fragment on bovine capillary endothelial cells.

Fig. 5 shows the identification of the 10 kDa fragment as kringle 4 by amino acid microsequencing.

Fig. 6 shows SEQ ID NO:1, the amino acid sequence of the whole murine plasminogen.

Detailed Description

The present invention includes compositions and methods for the detection and treatment of diseases and processes that are mediated by or associated with angiogenesis. The composition is an angiostatin kringle 4 region, which can be isolated from body fluids including, but not limited to, serum, urine and ascites, or synthesized by chemical or biological methods (e.g. cell culture, recombinant gene expression, protein synthesis, and *in vitro* enzymatic catalysis of angiostatin, plasminogen or plasmin to yield active kringle 4 region peptides). Recombinant techniques include gene amplification from DNA sources using the polymerase chain reaction (PCR), and gene amplification from RNA sources using reverse transcriptase/PCR. These angiostatin kringle 4 region fragments inhibit the growth of blood vessels into tissues such as de-vascularized or vascularized tumors.

The description of angiostatin and other kringle 4 region fragments can be found, for example, in U.S. Patent Nos. 5,639,725; 5,733,876 and 5,837,682, the entire contents of which are hereby incorporated by reference.

The present invention also encompasses a composition comprising, a vector containing a DNA sequence encoding angiostatin kringle 4 region fragments, wherein the vector is capable of expressing angiostatin kringle 4 region fragments when present in a cell, and a method comprising, implanting into a human or non-human animal a cell containing a vector, wherein the vector contains a DNA sequence encoding kringle 4 region fragments, and wherein the vector is capable of expressing kringle 4 region fragments when present in the cell. The cell may contain one vector or multiple vectors.

Still further, the present invention encompasses kringle 4 region fragments, kringle 4 region antisera, kringle 4 region receptor agonists or kringle 4 region receptor antagonists that are combined with pharmaceutically acceptable excipients, and optionally sustained-release compounds or compositions, such as biodegradable polymers, to form therapeutic compositions. In particular, the invention includes a composition comprising an antibody that specifically binds to a kringle 4 region, wherein the antibody does not bind to plasminogen.

More particularly, the present invention includes a protein designated angiostatin kringle 4 region that has a molecular weight of approximately 10 kilodaltons (kDa) as determined by reducing polyacrylamide gel electrophoresis that is capable of overcoming the angiogenic activity of endogenous growth factors such as bFGF, *in vitro*. Kringle 4 is typically defined as encompassing amino acids 377-454 of a human plasminogen molecule. (The amino acid sequence of the complete murine plasminogen molecule is shown in Figure 6 and in SEQ ID NO:1.) However, the kringle 4 region is surrounded by inter-kringle domains on either end, portions of which may be included in functional kringle 4 region fragments of the present

invention. For example, functional murine kringle 4 region fragments have been demonstrated herein to have anti-endothelial cell proliferation activity encompassing amino acids 371-458, 374-458, and 376-458. Therefore, it should be understood that the term "region" encompasses all such anti-angiogenic kringle 4 fragments containing varying numbers of amino acids from the amino and carboxy terminal inter-kringle domains. It is also to be understood that the present invention is contemplated to include any derivatives of the angiostatin kringle 4 region fragment that have endothelial inhibitory activity. These include proteins with angiostatin kringle 4 region activity that have amino acid substitutions or have sugars or other molecules attached to amino acid functional groups.

The term "substantially similar," when used in reference to angiostatin kringle 4 region fragment amino acid sequences, means an amino acid sequence having anti-angiogenic activity, which also has a high degree of sequence homology to the human protein fragment of kringle 4 fragments. A high degree of homology means at least approximately 60% amino acid homology, desirably at least approximately 70% amino acid homology, and more desirably at least approximately 80% amino acid homology. Homology is often measured using sequence analysis software, e.g., BLASTIN or BLASTP (available at http://www.ncbi.nlm.nih.gov/BLAST). The default parameters for comparing the two sequences (e.g., "Blast"-ing two sequences against each other) by BLASTIN (for nucleotide sequences) are reward for match =1, penalty for mismatch = -2, open gap = 5, and extension gap = 2. When using BLASTP for protein sequences, the default parameters are reward for match = 0, penalty for mismatch = 0, open gap = 11, and extension gap = 1.

The term "endothelial inhibiting activity" as used herein means the capability of a molecule to inhibit angiogenesis in general and, for example, to inhibit the growth of bovine

capillary endothelial cells in culture in the presence of fibroblast growth factor.

The kringle 4 region of angiostatin has been shown to be capable of inhibiting the growth of endothelial cells *in vitro*. Angiostatin kringle 4 region does not inhibit the growth of cell lines derived from other cell types. Specifically, angiostatin kringle 4 region has no effect on Lewis lung carcinoma cell lines, mink lung epithelium, 3T3 fibroblasts, bovine aortic smooth muscle cells, bovine retinal pigment epithelium, MDCk cells (canine renal epithelium), WI38 cells (human fetal lung fibroblasts) EFN cells (murine fetal fibroblasts) and LM cells (murine connective tissue). Endogenous angiostatin in a tumor bearing mouse is effective at inhibiting metastases at a systemic concentration of approximately 10 mg angiostatin/kg body weight.

Angiostatin has a specific three dimensional conformation that is defined by the kringle regions of the plasminogen molecule. (Robbins, K.C., "The plasminogen-plasmin enzyme system" Hemostasis and Thrombosis, Basic Principles and Practice, 2nd Edition, ed. by Colman, R.W. et al. J.B. Lippincott Company, pp. 340-357, 1987) There are five such kringle regions, which are conformationally related motifs and have substantial sequence homology, in the NH_2 terminal portion of the plasminogen molecule. Each kringle region of the plasminogen molecule contains approximately 80 amino acids and contains 3 disulfide bonds. This cysteine motif is known to exist in other biologically active proteins. These proteins include, but are not limited to, prothrombin, hepatocyte growth factor, scatter factor and macrophage stimulating protein. (Yoshimura, T, et al., "Cloning, sequencing, and expression of human macrophage stimulating protein (MSP, MST1) confirms MSP as a member of the family of kringle proteins and locates the MSP gene on Chromosome 3" *J. Biol. Chem.*, Vol. 268, No. 21, pp. 15461-15468, 1993). It is contemplated that any isolated kringle 4 region fragment having a three dimensional kringle-like

conformation or cysteine motif that has anti-angiogenic activity *in vivo*, is part of the present invention.

The present invention also includes the detection of the angiostatin kringle 4 region fragments in body fluids and tissues for the purpose of diagnosis or prognosis of diseases such as cancer. The present invention also includes the detection of angiostatin kringle 4 region fragment binding sites and receptors in cells and tissues. The present invention also includes methods of treating or preventing angiogenic diseases and processes including, but not limited to, arthritis and tumors by stimulating the production of angiostatin kringle 4 region fragments, and/or by administering substantially purified angiostatin kringle 4 region fragments, nucleotides encoding angiostatin kringle 4 region fragments, or angiostatin kringle 4 region fragment agonists or antagonists, and/or angiostatin kringle 4 region fragment antisera or antisera directed against angiostatin kringle 4 region fragment antisera to a patient. Additional treatment methods include administration of angiostatin kringle 4 region fragments, angiostatin kringle 4 region fragment analogs, angiostatin kringle 4 region fragment antisera, or angiostatin receptor agonists and antagonists linked to cytotoxic agents. It is to be understood that the angiostatin kringle 4 region fragments can be animal or human in origin. Angiostatin kringle 4 region fragments can be produced synthetically by chemical reaction or by recombinant techniques in conjunction with expression systems. Angiostatin kringle 4 region fragments may also be produced *in vitro* or *in vivo* by enzymatically cleaving angiostatin, plasminogen or plasmin to generate proteins having anti-angiogenic activity or by using compounds that mimic the action of endogenous enzymes that cleave angiostatin or plasminogen into kringle 4 region fragments. Angiostatin kringle 4 region fragment production may also be modulated by compounds that affect the activity of plasminogen cleaving enzymes.

Passive antibody therapy using antibodies that specifically bind angiostatin kringle 4 region fragments can be employed to modulate angiogenic-dependent processes such as reproduction, development, and wound healing and tissue repair. In addition, antisera directed to the Fab regions of angiostatin kringle 4 region fragment antibodies can be administered to block the ability of endogenous angiostatin kringle 4 region fragment antisera to bind angiostatin kringle 4 region fragments.

The present invention also encompasses gene therapy whereby the gene encoding an angiostatin kringle 4 region fragment is regulated in a patient. Various methods of transferring or delivering DNA to cells for expression of the gene product protein, otherwise referred to as gene therapy, are disclosed in Gene Transfer into Mammalian Somatic Cells *in vivo,* N. Yang, Crit. Rev. Biotechn. 12(4): 335-356 (1992), which is hereby incorporated by reference. Gene therapy encompasses incorporation of DNA sequences into somatic cells or germ line cells for use in either *ex vivo* or *in vivo* therapy. Gene therapy functions to replace genes, augment normal or abnormal gene function, and to combat infectious diseases and other pathologies.

Strategies for treating these medical problems with gene therapy include therapeutic strategies such as identifying the defective gene and then adding a functional gene to either replace the function of the defective gene or to augment a slightly functional gene; or prophylactic strategies, such as adding a gene encoding the protein product that will treat the condition or that will make the tissue or organ more susceptible to a treatment regimen. As an example of a prophylactic strategy, a gene for an angiostatin kringle 4 region fragment may be placed in a patient and thus prevent occurrence of angiogenesis; or a gene that makes tumor cells more susceptible to radiation could be inserted and then radiation of the tumor would cause increased killing of the tumor cells.

Many protocols for transfer of angiostatin kringle 4 region fragment DNA or angiostatin kringle 4 region fragment regulatory sequences are envisioned in this invention. Transfection of promoter sequences, other than one normally found specifically associated with angiostatin, or other sequences which would increase production of angiostatin kringle 4 region proteins are also envisioned as methods of gene therapy. An example of this technology is found in Transkaryotic Therapies, Inc., of Cambridge, Massachusetts, using homologous recombination to insert a "genetic switch" that turns on an erythropoietin gene in cells. See Genetic Engineering News, April 15, 1994. Such "genetic switches" could be used to activate an angiostatin kringle 4 region fragment (or the angiostatin kringle 4 region fragment receptor) in cells not normally expressing angiostatin kringle 4 region fragment (or the angiostatin kringle 4 region fragment receptor).

Gene transfer methods for gene therapy fall into three broad categories: (1) physical (e.g., electroporation, direct gene transfer and particle bombardment), (2) chemical (lipid-based carriers, or other non-viral vectors) and (3) biological (virus-derived vector and receptor uptake). For example, non-viral vectors may be used which include liposomes coated with DNA. Such liposome/DNA complexes may be directly injected intravenously into the patient. It is believed that the liposome/DNA complexes are concentrated in the liver where they deliver the DNA to macrophages and Kupffer cells. These cells are long lived and thus provide long term expression of the delivered DNA. Additionally, vectors or the "naked" DNA of the gene may be directly injected into the desired organ, tissue or tumor for targeted delivery of the therapeutic DNA.

Gene therapy methodologies can also be described by delivery site. Fundamental ways to deliver genes include *ex vivo* gene transfer, *in vivo* gene transfer, and *in vitro* gene transfer. In *ex vivo* gene transfer, cells are taken from the patient and grown in cell culture. The DNA is transfected into the cells, the

transfected cells are expanded in number and then re-implanted in the patient. In *in vitro* gene transfer, the transformed cells are cells growing in culture, such as tissue culture cells, and not particular cells from a particular patient. These "laboratory cells" are transfected, the transfected cells are selected and expanded for either implantation into a patient or for other uses.

In vivo gene transfer involves introducing the DNA into the cells of the patient when the cells are within the patient. Methods include using virally mediated gene transfer using a noninfectious virus to deliver the gene in the patient or injecting naked DNA into a site in the patient and the DNA is taken up by a percentage of cells in which the gene product protein is expressed. Additionally, the other methods described herein, such as use of a "gene gun," may be used for *in vitro* insertion of angiostatin kringle 4 region fragment DNA or angiostatin regulatory sequences.

Chemical methods of gene therapy may involve a lipid based compound, not necessarily a liposome, used to ferry the DNA across the cell membrane. Lipofectins or cytofectins, lipid-based positive ions that bind to negatively charged DNA, make a complex that can cross the cell membrane and provide the DNA into the interior of the cell. Biological methods used in gene therapy techniques may involve receptor-based endocytosis, or receptor-based phagocytosis, which involve binding a specific ligand to a cell surface receptor and enveloping and transporting the ligand across the cell membrane. Specifically, a ligand gene complex is created and injected into the blood stream and then target cells that have the receptor will specifically bind the ligand and transport the ligand-DNA complex into the cell.

Many gene therapy methodologies employ viral vectors to insert genes into cells. For example, altered retrovirus vectors have been used in *ex vivo* methods to introduce genes into peripheral and tumor-infiltrating lymphocytes, hepatocytes, epidermal cells, myocytes, and other somatic cells. These altered

cells are then introduced into the patient to provide the gene product from the inserted DNA.

Viral vectors have also been used to insert genes into cells using *in vivo* protocols. To accomplish tissue-specific expression of foreign genes, cis-acting regulatory elements or promoters that are known to be tissue specific can be used. Alternatively, tissue-specific expression can be achieved using *in situ* delivery of DNA or viral vectors to specific anatomical sites *in vivo*. For example, gene transfer to blood vessels *in vivo* was achieved by implanting *in vitro* transduced endothelial cells in chosen sites on arterial walls. The virus infected surrounding cells which also expressed the gene product. A viral vector can be delivered directly to the *in vivo* site, by a catheter for example, thus allowing only certain areas to be infected by the virus, and providing long-term, site specific gene expression. *In vivo* gene transfer using retrovirus vectors has also been demonstrated in mammary tissue and hepatic tissue by injection of the altered virus into blood vessels leading to the organs.

Viral vectors that have been used for gene therapy protocols include but are not limited to, retroviruses, other RNA viruses such as poliovirus or Sindbis virus , adenovirus, adeno-associated virus, herpes viruses, SV 40, vaccinia and other DNA viruses. Replication-defective murine retroviral vectors are the most widely utilized gene transfer vectors. Murine leukemia retroviruses are composed of a single strand RNA complexed with a nuclear core protein and polymerase (pol) enzymes, encased by a protein core (gag) and surrounded by a glycoprotein envelope (env) that determines host range. The genomic structure of retroviruses includes the gag, pol, and env genes flanked by 5' and 3' long terminal repeats (LTR). Retroviral vector systems exploit the fact that a minimal vector containing the 5' and 3' LTRs and the packaging signal are sufficient to allow vector packaging, infection and integration into target cells providing that the viral structural proteins are supplied *in trans* in the packaging cell line. Fundamental

advantages of retroviral vectors for gene transfer include efficient infection and gene expression in most cell types, precise single copy vector integration into target cell chromosomal DNA, and ease of manipulation of the retroviral genome.

The adenovirus is composed of linear, double stranded DNA complexed with core proteins and surrounded with capsid proteins. Advances in molecular virology have led to the ability to exploit the biology of these organisms to create vectors capable of transducing novel genetic sequences into target cells *in vivo*. Adenoviral-based vectors will express gene product proteins at high levels. Adenoviral vectors have high efficiencies of infectivity, even with low titers of virus. Additionally, the virus is fully infective as a cell free virion so injection of expression cell lines is not necessary. Another potential advantage to adenoviral vectors is the ability to achieve long term expression of heterologous genes *in vivo*.

Mechanical methods of DNA delivery include fusogenic lipid vesicles such as liposomes or other vesicles for membrane fusion, lipid particles of DNA incorporating cationic lipids such as lipofectin, polylysine-mediated transfer of DNA, direct injection of DNA, such as microinjection of DNA into germ or somatic cells, pneumatically delivered DNA-coated particles, such as the gold particles used in a "gene gun," and inorganic chemical approaches such as calcium phosphate transfection.

It has been found that injecting plasmid DNA into muscle cells yields high percentage of the cells which are transfected and have sustained expression of marker genes. The DNA of the plasmid may or may not integrate into the genome of the cells. Non-integration of the transfected DNA would allow the transfection and expression of gene product proteins in terminally differentiated, non-proliferative tissues for a prolonged period of time without fear of mutational insertions, deletions, or alterations in the cellular or mitochondrial genome. Long-term, but not necessarily permanent, transfer of therapeutic genes into specific cells may provide treatments for genetic diseases or for

prophylactic use. The DNA could be re-injected periodically to maintain the gene product level without mutations occurring in the genomes of the recipient cells. Non-integration of exogenous DNAs may allow for the presence of several different exogenous DNA constructs within one cell with all of the constructs expressing various gene products.

Particle-mediated gene transfer methods were first used in transforming plant tissue. With a particle bombardment device, or "gene gun," a motive force is generated to accelerate DNA-coated high density particles (such as gold or tungsten) to a high velocity that allows penetration of the target organs, tissues or cells. Particle bombardment can be used in *in vitro* systems, or with *ex vivo* or *in vivo* techniques to introduce DNA into cells, tissues or organs.

Electroporation for gene transfer uses an electrical current to make cells or tissues susceptible to electroporation-mediated gene transfer. A brief electric impulse with a given field strength is used to increase the permeability of a membrane in such a way that DNA molecules can penetrate into the cells. This technique can be used in *in vitro* systems, or with *ex vivo* or *in vivo* techniques to introduce DNA into cells, tissues or organs.

Carrier mediated gene transfer *in vivo* can be used to transfect foreign DNA into cells. The carrier-DNA complex can be conveniently introduced into body fluids or the bloodstream and then site specifically directed to the target organ or tissue in the body. Both liposomes and polycations, such as polylysine, lipofectins or cytofectins, can be used. Liposomes can be developed which are cell specific or organ specific and thus the foreign DNA carried by the liposome will be taken up by target cells. Injection of immunoliposomes that are targeted to a specific receptor on certain cells can be used as a convenient method of inserting the DNA into the cells bearing the receptor. Another carrier system that has been used is the asialoglycoportein/polylysine conjugate system for carrying DNA to hepatocytes for *in vivo* gene transfer.

The transfected DNA may also be complexed with other kinds of carriers so that the DNA is carried to the recipient cell and then resides in the cytoplasm or in the nucleoplasm. DNA can be coupled to carrier nuclear proteins in specifically engineered vesicle complexes and carried directly into the nucleus.

Gene regulation of angiostatin kringle 4 region fragment may be accomplished by administering compounds that bind to the angiostatin gene, or control regions associated with the angiostatin gene, or its corresponding RNA transcript to modify the rate of transcription or translation. Additionally, cells transfected with a DNA sequence encoding angiostatin kringle 4 region fragment may be administered to a patient to provide an *in vivo* source of angiostatin. For example, cells may be transfected with a vector containing a nucleic acid sequence encoding angiostatin. The term "vector" as used herein means a carrier that can contain or associate with specific nucleic acid sequences, which functions to transport the specific nucleic acid sequences into a cell. Examples of vectors include plasmids and infective microorganisms such as viruses, or non-viral vectors such as ligand-DNA conjugates, liposomes, lipid-DNA complexes. It may be desirable that a recombinant DNA molecule comprising a kringle 4 region DNA sequence is operatively linked to an expression control sequence to form an expression vector capable of expressing kringle 4 region fragments. The transfected cells may be cells derived from the patient's normal tissue, the patient's diseased tissue, or may be non-patient cells.

For example, tumor cells removed from a patient can be transfected with a vector capable of expressing the angiostatin kringle 4 region fragment of the present invention, and re-introduced into the patient. The transfected tumor cells produce angiostatin kringle 4 region fragment at levels that inhibit the growth of the tumor. Patients may be human or non-human animals. Cells may also be transfected by non-vector, or

physical or chemical methods known in the art such as electroporation, ionoporation, or via a "gene gun." Additionally, angiostatin kringle 4 region fragment DNA may be directly injected, without the aid of a carrier, into a patient. In particular, angiostatin kringle 4 region fragment DNA may be injected into skin, muscle or blood.

The gene therapy protocol for transfecting angiostatin kringle 4 region fragments into a patient may either be through integration of the angiostatin DNA into the genome of the cells, into minichromosomes or as a separate replicating or non-replicating DNA construct in the cytoplasm or nucleoplasm of the cell. Angiostatin kringle 4 region fragment expression may continue for a long-period of time or may be re-injected periodically to maintain a desired level of the angiostatin kringle 4 region fragment protein in the cell, the tissue or organ or a determined blood level.

One example of a method of producing angiostatin kringle 4 region fragments using recombinant DNA techniques entails the steps more fully described in laboratory manuals such as "Molecular Cloning: A Laboratory Manual" Second Edition by Sambrook et al., Cold Spring Harbor Press, 1989. The DNA sequence of human plasminogen has been published (Browne, M. J., et al., "Expression of recombinant human plasminogen and aglycoplasminogen in HeLa cells" Fibrinolysis Vol. 5 (4). 257-260, 1991) and is incorporated herein by reference

The fragment can also be synthesized by techniques well known in the art, as exemplified by "Solid Phase Protein Synthesis: A Practical Approach" E. Atherton and R.C. Sheppard, IRL Press, Oxford, England. Similarly, multiple fragments can be synthesized which are subsequently linked together to form larger fragments. These synthetic protein fragments can also be made with amino acid substitutions at specific locations to test for agonistic and antagonistic activity *in vitro* and *in vivo*. Protein fragments that possess high affinity binding to tissues can be used to isolate the angiostatin kringle 4

region fragment receptor on affinity columns. Isolation and purification of the angiostatin kringle 4 region fragment receptor is a fundamental step towards elucidating the mechanism of action of angiostatin kringle 4 regions. Isolation of an angiostatin kringle 4 region fragment receptor and identification of agonists and antagonists of that receptor will facilitate development of drugs to modulate the activity of the angiostatin kringle 4 region fragment receptor. Isolation of the receptor enables the construction of nucleotide probes to monitor the location and synthesis of the receptor, using *in situ* and solution hybridization technology. Further, the gene for the receptor can be isolated, incorporated into an expression vector and transfected into cells, such as patient tumor cells to increase the ability of a cell type, tissue or tumor to bind angiostatin kringle 4 region fragments and inhibit local angiogenesis.

An angiostatin kringle 4 region fragment is effective in treating diseases or processes that are mediated by, or involve, angiogenesis. The present invention includes the method of treating an angiogenesis mediated disease with an effective amount of angiostatin kringle 4 region fragment, or combinations of kringle 4 region fragments that collectively possess anti-angiogenic activity, or angiostatin kringle 4 region agonists and antagonists. The angiogenesis mediated diseases include, but are not limited to, solid tumors; blood born tumors such as leukemias; tumor metastasis; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation. Angiostatin is useful in the treatment of disease of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to,

intestinal adhesions, Crohn's disease, atherosclerosis, scleroderma, and hypertrophic scars, i.e., keloids. Angiostatin kringle 4 region fragment can be used as a birth control agent by preventing vascularization required for embryo implantation. Angiostatin kringle 4 region fragment is useful in the treatment of diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (*Rochele minalia quintosa*) and ulcers (*Helicobacter pylori*).

Angiostatin kringle 4 region fragments may be used in combination with other compositions and procedures for the treatment of diseases. For example, a tumor may be treated conventionally with surgery, radiation or chemotherapy combined with angiostatin kringle 4 region fragments and then angiostatin kringle 4 region fragments may be subsequently administered to the patient to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tumor. Additionally, angiostatin kringle 4 region fragments, angiostatin kringle 4 region antisera, angiostatin kringle 4 region receptor agonists or antagonists, or combinations thereof, are combined with pharmaceutically acceptable excipients, and optionally a sustained-release matrix, such as biodegradable polymers, to form therapeutic compositions.

The angiogenesis-modulating therapeutic composition of the present invention may be a solid, liquid or aerosol and may be administered by any known route of administration. Examples of solid therapeutic compositions include pills, creams, and implantable dosage units. The pills may be administered orally, the therapeutic creams may be administered topically. The implantable dosage units may be administered locally, for example at a tumor site, or which may be implanted for systemic release of the therapeutic angiogenesis-modulating composition, for example subcutaneously. Examples of liquid composition include formulations adapted for injection subcutaneously, intravenously, intraarterially, and formulations for topical and

intraocular administration. Examples of aersol formulations include inhaler formulations for administration to the lungs.

The angiostatin kringle 4 region fragments of the present invention also can be used to generate antibodies that are specific for the inhibitor and its receptor. The antibodies can be either polyclonal antibodies or monoclonal antibodies. To enhance the potential for high specificity in the development of antisera, (or agonists and antagonists) to angiostatin, protein sequences can be compared to known sequences using protein sequence databases such as GenBank, Brookhaven Protein, SWISS-PROT, and PIR to determine potential sequence homologies. This information facilitates elimination of sequences that exhibit a high degree of sequence homology to other molecules. These antibodies that specifically bind to the angiostatin kringle 4 region fragment or their receptors, can be used in diagnostic methods and kits that are well known to those of ordinary skill in the art to detect or quantify the angiostatin kringle 4 region fragments or receptors in a body fluid or tissue. Results from these tests can be used to diagnose or predict the occurrence or recurrence of a cancer or other angiogenic mediated disease.

Another aspect of the present invention is a method of blocking the action of excess endogenous angiostatin kringle 4 region fragments. This can be done by passively immunizing a human or animal with antibodies specific for the undesired angiostatin kringle 4 region fragment in the system. This treatment can be important in treating abnormal ovulation, menstruation and placentation, and vasculogenesis. This provides a useful tool to examine the effects of angiostatin kringle 4 region fragment removal on metastatic processes. The Fab fragment of angiostatin kringle 4 region fragment antibodies contains the binding site for angiostatin kringle 4 region fragment. This fragment is isolated from antibodies using techniques known to those skilled in the art. The Fab fragments of angiostatin kringle 4 region fragment antisera are then used as antigens to generate production of anti-Fab fragment serum.

Infusion of anti-Fab fragment serum prevents angiostatin kringle 4 region fragments from binding to endogenous antibodies. The net effect of this treatment is to facilitate the ability of endogenous circulating angiostatin kringle 4 region fragment to reach target cells, thereby decreasing the spread of metastases.

The proteins and protein fragments with the angiostatin kringle 4 region fragment activity described above can be provided as isolated and substantially purified proteins and protein fragments in pharmaceutically acceptable formulations using formulation methods known to those of ordinary skill in the art. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (e.g., intravenous, intraspinal, subcutaneous or intramuscular) route. In addition, the angiostatin kringle 4 region fragment may be incorporated into biodegradable polymers allowing for sustained release of the compound, the polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the angiostatin is slowly released systemically. The biodegradable polymers and their use are described, for example, in detail in Brem et al., *J. Neurosurg.* 74:441-446 (1991), which is hereby incorporated by reference in its entirety. Osmotic minipumps may also be used to provide controlled delivery of high concentrations of angiostatin kringle 4 region fragment through cannulae to the site of interest, such as directly into a metastatic growth or into the vascular supply to that tumor.

The dosage of the angiostatin kringle 4 region fragment of the present invention will depend on the disease state or condition being treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. For treating humans or animals, between approximately 0.5 mg/kilogram to 500

mg/kilogram of the angiostatin kringle 4 region fragment can be administered. Depending upon the half-life of the angiostatin kringle 4 region fragment in the particular animal or human, it can be administered between several times per day to once a week. It is to be understood that the present invention has application for both human and veterinary use. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time.

The angiostatin formulations may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules or vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients, particularly mentioned above,

the formulations of the present invention may include other agents conventional in the art having regard to the type of formulation in question. Optionally, cytotoxic agents may be incorporated or otherwise combined with angiostatin kringle 4 region fragment proteins, or biologically functional protein fragments thereof, to provide dual therapy to the patient.

Kits for measurement of angiostatin kringle 4 region fragment, and the receptor, are also contemplated as part of the present invention. Antisera that possess the highest titer and specificity and can detect angiostatin kringle 4 region fragment proteins in extracts of plasma, urine, tissues, and in cell culture media are further examined to establish easy to use kits for rapid, reliable, sensitive, and specific measurement and localization of angiostatin kringle 4 region fragments. These assay kits include but are not limited to the following techniques; competitive and non-competitive assays, radioimmunoassay, bioluminescence and chemiluminescence assays, fluorometric assays, sandwich assays, immunoradiometric assays, dot blots, enzyme linked assays including ELISA, antibody coated strips or dipsticks for rapid monitoring of urine or blood, and immunocytochemistry. For each kit the range, sensitivity, precision, reliability, specificity and reproducibility of the assay are established. Intra-assay and inter-assay variation is established at 20%, 50% and 80% points on the standard curves of displacement or activity.

In one embodiment of the present invention, a kit is used for localization of angiostatin kringle 4 region fragments in tissues and cells. This angiostatin immunohistochemistry kit provides instructions, angiostatin kringle 4 region fragment antiserum, and possibly blocking serum and secondary antiserum linked to a fluorescent molecule such as fluorescein isothiocyanate, or to some other reagent used to visualize the primary antiserum. Immunohistochemistry techniques are well known to those skilled in the art.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

Example 1

Characterization of Endothelial Cell Proliferation Inhibiting Kringle 4 region fragments

Recombinant murine angiostatin protein (Figure 1) purified from a lysine column as previously described (BBRC, 236:651, 1997) was concentrated to 0.2 ml with Centricon-10 concentrators and applied to a Sephadex G-75 column (46 cm x 1.5 cm) equilibrated with Phosphate Buffered Saline (PBS). The column was eluted with PBS and 1 ml aliquots were collected. A single peak of angiostatin protein which appeared as a 52 kDa band on SDS gel stained with ISS Pro-Blue was obtained (Figure 3, lane 2).

This 52 kDa angiostatin protein was left at 4°C for at least seven days. At the end of incubation, the angiostatin protein sample was analyzed on a Sephadex G-75 column in an identical manner (Figure 2). Two protein peaks were discovered. The first peak has a molecular weight of about 37 kDa (Figure 3, lane 4) and the second peak has a molecular weight of about 10 kDa (Figure 3, lane 5) as analyzed by SDS gel electrophoresis stained with ISS Pro-Blue. In Figure 3, lane 2 and 3 correspond to angiostatin samples before and after, respectively, the 4°C incubation. The 52 kDa angiostatin had no effect on DNA synthesis (Figure 3, lane 2) compared to PBS (Figure 3, lane 1). However, both the 37 kDa and the 10 kDa fragments inhibit DNA synthesis of bovine capillary endothelial

(BCE) cells (Figure 3). The 10 kDa fragment was also demonstrated to inhibit BCE cell DNA synthesis (Figure 4, upper panel) and proliferation (Figure 4, lower panel) in a dose-dependent manner. Amino acid sequence analysis of the 10 kDa fragment reveals that it consists of a mixture of three different forms of Kringle 4 of plasminogen (AA_{377} - AA_{454}), and the variability in the sequences is attributable to the point of cleavage between Kringle 3 and 4 (Figure 5).

It should be understood that the foregoing relates only to preferred embodiments of the present invention, and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the invention as set forth in the appended claims.

Claims

We claim:

1. A method of inhibiting angiogenesis in an individual comprising, increasing in the individual *in vivo* concentrations of a kringle 4 region fragment of a plasminogen molecule to an angiogenesis inhibiting amount, wherein the kringle 4 region fragment has anti-angiogenic activity.

2. The method of Claim 1, wherein the kringle 4 region fragment is administered to the individual.

3. The method of Claim 1, wherein a nucleic acid encoding the kringle 4 region fragment is administered to the individual.

4. The method of Claim 1, wherein the kringle 4 region fragment is derived from murine plasminogen, human plasminogen, Rhesus plasminogen, porcine plasminogen or bovine plasminogen.

5. The method of Claim 1, wherein the kringle 4 region fragment has an amino acid sequence selected from the group consisting of amino acids 371-458, 374-458 and 376-458 of a human plasminogen molecule.

6. The method of Claim 1, wherein the kringle 4 region fragment has anti-angiogenic activity *in vivo*.

7. The method of Claim 1, wherein the kringle 4 region fragment has anti-angiogenic activity *in vitro*.

8. A method of treating a mammal with an angiogenic-mediated disease comprising, administering to the mammal a treatment effective amount of a kringle 4 region

fragment of a plasminogen molecule, wherein the kringle 4 region fragment has anti-angiogenic activity..

9. The method of Claim 8, wherein the mammal is a human.

10. The method of Claim 8, wherein the angiogenic mediated disease is selected from the group consisting of cancer, arthritis, macular degeneration and diabetic retinopathy.

11. The method of Claim 8, wherein the kringle 4 region fragment has an amino acid sequence selected from the group consisting of amino acids 371-458, 374-458 and 376-458 of a human plasminogen molecule.

12. A therapeutic composition for inhibiting angiogenesis comprising a substantially isolated kringle 4 region fragment of a plasminogen molecule and a pharmaceutically acceptable excipient.

13. The composition of Claim 12, wherein the kringle 4 region fragment is derived from murine plasminogen, human plasminogen, Rhesus plasminogen, porcine plasminogen or bovine plasminogen.

14. The composition of Claim 12, wherein the kringle 4 region fragment has an amino acid sequence selected from the group consisting of amino acids 371-458, 374-458 and 376-458 of a human plasminogen molecule.

15. A composition comprising, an isolated DNA sequence that codes for a kringle 4 region fragment of a plasminogen molecule, wherein the kringle 4 region fragment has anti-angiogenic activity.

16. The composition of Claim 15, wherein the DNA sequence codes for a kringle 4 region fragment having an amino acid sequence selected from the group consisting of amino acids 371-458, 374-458 and 376-458 of a human plasminogen molecule.

17. The composition of Claim 15, further comprising a vector associated with the DNA sequence encoding the kringle 4 region, wherein the vector is capable of expressing the kringle 4 region fragment when present in a cell.

18. The composition of Claim 17, further comprising a cell containing said vector.

19. A method of expressing a kringle 4 region fragment of a plasminogen having an endothelial cell proliferation inhibiting activity comprising, transfecting in a mammalian cell a vector, wherein the vector contains a DNA sequence encoding the kringle 4 region fragment, and wherein the vector is capable of expressing the kringle 4 region fragment when present in the cell.

20. The method of Claim 19, wherein the DNA sequence codes for a kringle 4 region fragment having an amino acid sequence selected from the group consisting of amino acids 371-458, 374-458 and 376-458 of a human plasminogen molecule

Production of Recombinant Murine Angiostatin with a Baculovirus Expression System

Mouse Plasminogen

1----------19--------98----------458----------812 aa
Signal peptide Kringle 1 Kringle 2 Kringle 3 Kringle 4 Kringle 5

Angiostatin gene designed for transfection

1----32,98----------458 aa

↓

BaculoGold® + Transfer vector → Insect cells
(Modified baculovirus) (pVL1393)

↓

Recombinant Murine Angiostatin

Figure 1.

Gel filtration chromatography of angiostatin degradation products

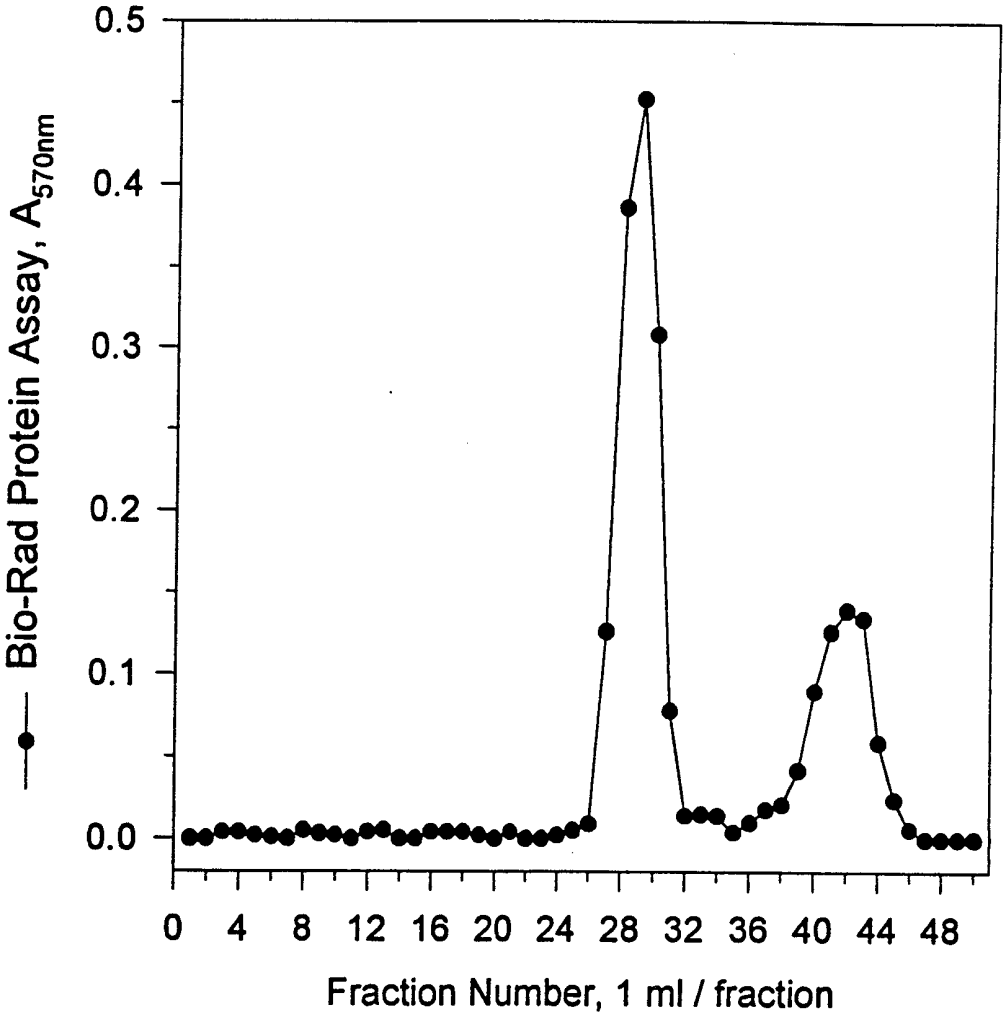


Figure 2.

Production of fragments from 52K recombinant murine angiostatin

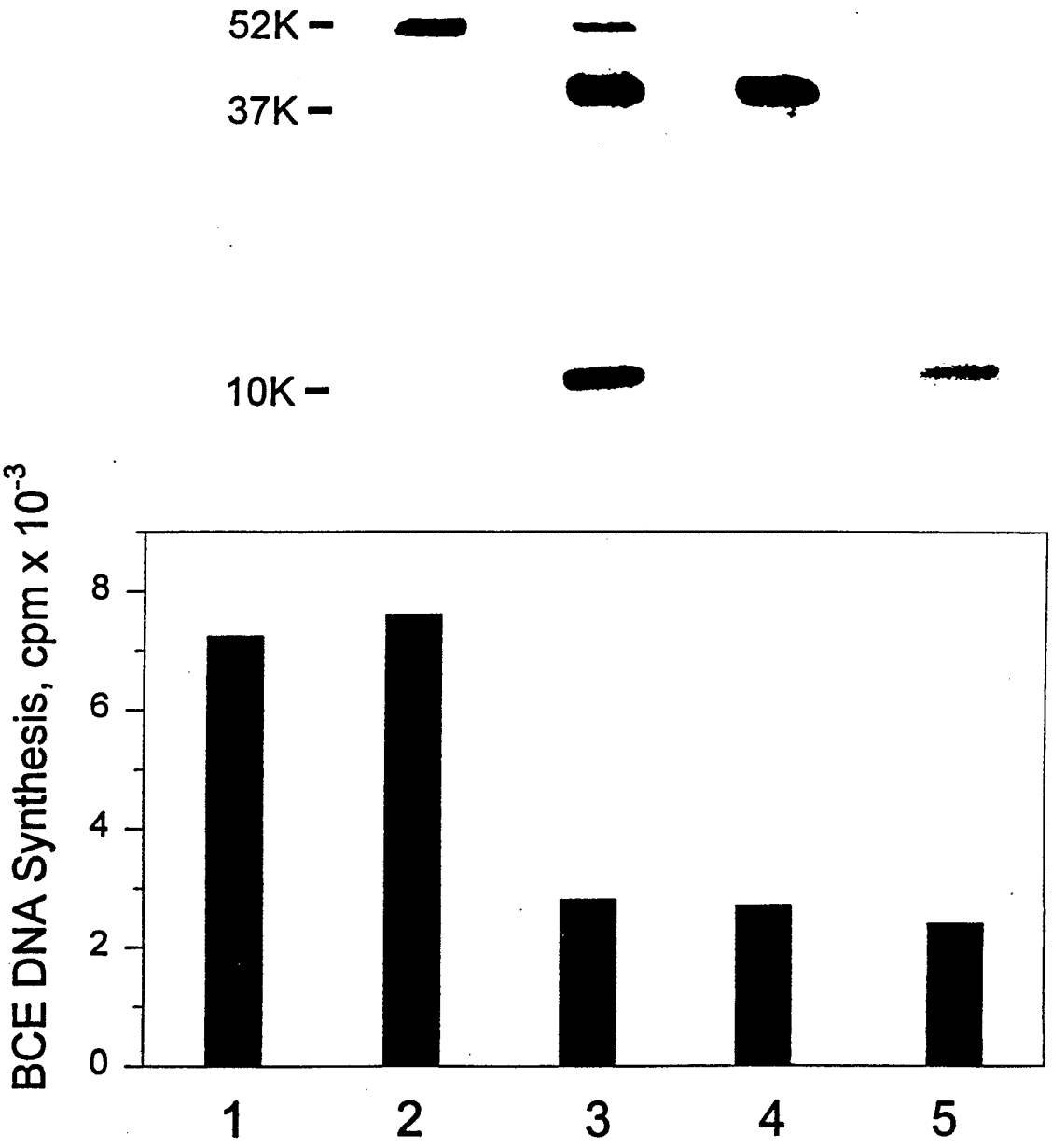


Figure 3.

Inhibitory effects of 10K fragment on bovine capillary endothelial cells

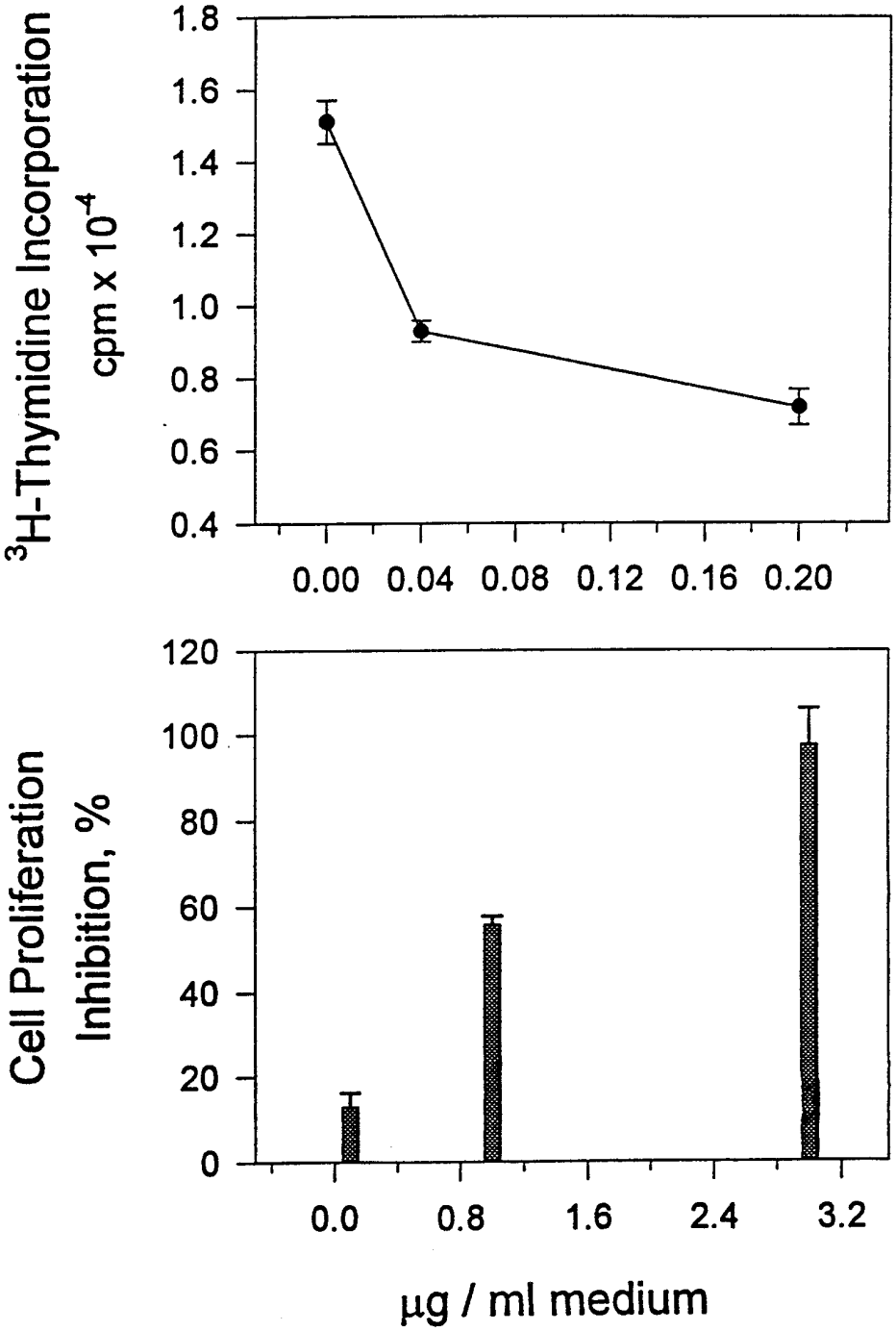


Figure 4.

Identification of the 10K fragment as kringle 4 by amino acid microsequencing

N-terminal Sequencing Data

Cycle:	1	2	3	4	5	6	7	8	9
	V	Q	Y	Q	I	D	I	D	S
	T	P	E	V	Y	Q	G	Y	G
	E		V	C	S	E			Q

(Amino acids found)

Data Analysis

Angiostatin aa: 371 374 376

Q-T-P-V-V-Q-E-C-Y-Q-S-D-G-Q-S-

↑ ↑ ↑

Cleavage site: 1 2 3

10K fragments

Cleavage 1 → T-P-V-V-Q-E-C-Y-Q → AA_{371} to AA_{458}

Cleavage 2 → V-Q-E-C-Y-Q-S-D-G → AA_{374} to AA_{458}

Cleavage 3 → E-C-Y-Q-S-D-G-Q-S → AA_{376} to AA_{458}

Figure 5.

Mouse Plasminogen Sequence:

met asp his lys glu val ile leu leu phe leu leu leu leu lys
pro gly gln gly asp ser leu asp gly tyr ile ser thr gln gly
ala ser leu phe ser leu thr lys lys gln leu ala ala gly gly
val ser asp cys leu ala lys cys glu gly glu thr asp phe val
cys arg ser phe gln tyr his ser lys glu gln gln cys val ile
met ala glu asn ser lys thr ser ser ile ile arg met arg asp
val ile leu phe glu lys arg val tyr leu ser glu cys lys thr
gly ile gly asn gly tyr arg gly thr met ser arg thr lys ser
gly val ala cys gln lys trp gly ala thr phe pro his val pro
asn tyr ser pro ser thr his pro asn glu gly leu glu glu asn
tyr cys arg asn pro asp asn asp glu gln gly pro trp cys tyr
thr thr asp pro asp lys arg tyr asp tyr cys asn ile pro glu
cys glu glu glu cys met tyr cys ser gly glu lys tyr glu gly
lys ile ser lys thr met ser gly leu asp cys gln ala trp asp
ser gln ser pro his ala his gly tyr ile pro ala lys phe pro
ser lys asn leu lys met asn tyr cys his asn pro asp gly glu
pro arg pro trp cys phe thr thr asp pro thr lys arg trp glu
tyr cys asp ile pro arg cys thr thr pro pro pro pro pro ser
pro thr tyr gln cys leu lys gly arg gly glu asn tyr arg gly
thr val ser val thr val ser gly lys thr cys gln arg trp ser
glu gln thr pro his arg his asn arg thr pro glu asn phe pro
cys lys asn leu glu glu asn tyr cys arg asn pro asp gly glu
thr ala pro trp cys tyr thr thr asp ser gln leu arg trp glu
tyr cys glu ile pro ser cys glu ser ser ala ser pro asp gln
ser asp ser ser val pro pro glu glu gln thr pro val val gln
glu cys tyr gln ser asp gly gln ser tyr arg gly thr ser ser
thr thr ile thr gly lys lys cys gln ser trp ala ala met phe
pro his arg his ser lys thr pro glu asn phe pro asp ala gly
leu glu met asn tyr cys arg asn pro asp gly asp lys gly pro
trp cys tyr thr thr asp pro ser val arg trp glu tyr cys asn
leu lys arg cys ser glu thr gly gly ser val val glu leu pro
thr val ser gln glu pro ser gly pro ser asp ser glu thr asp
cys met tyr gly asn gly lys asp tyr arg gly lys thr ala val
thr ala ala gly thr pro cys gln gly trp ala ala gln glu pro
his arg his ser ile phe thr pro gln thr asn pro arg ala asp
leu glu lys asn tyr cys arg asn pro asp gly asp val asn gly
pro trp cys tyr thr thr asn pro arg lys leu tyr asp tyr cys
asp ile pro leu cys ala ser ala ser ser phe glu cys gly lys
pro gln val glu pro lys lys cys pro gly arg val val gly gly
cys val ala asn pro his ser trp pro trp gln ile ser leu arg
thr arg phe thr gly gln his phe cys gly gly thr leu ile ala
pro glu trp val leu thr ala ala his cys leu glu lys ser ser
arg pro glu phe tyr lys val ile leu gly ala his glu glu tyr
ile arg gly leu asp val gln glu ile ser val ala lys leu ile
leu glu pro asn asn arg asp ile ala leu leu lys leu ser arg
pro ala thr ile thr asp lys val ile pro ala cys leu pro ser

Fig- 6A

pro asn tyr met val ala asp arg thr ile cys tyr ile thr gly
trp gly glu thr gln gly thr phe gly ala gly arg leu lys glu
ala gln leu pro val ile glu asn lys val cys asn arg val glu
tyr leu asn asn arg val lys ser thr glu leu cys ala gly gln
leu ala gly gly val asp ser cys gln gly asp ser gly gly pro
leu val cys phe glu lys asp lys tyr ile leu gln gly val thr
ser trp gly leu gly cys ala arg pro asn lys pro gly val tyr
val arg val ser arg phe val asp trp ile glu arg glu met arg
asn asn

Fig. 6B