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(54) Title: RIBOZYME THERAPY FOR RESTENOSIS

(57) Abstract

As an effective therapy for restenosis, this invention provides ribozymes and ribozyme delivery systems useful to inhibit abnormal smooth muscle cell proliferation in vascular tissue. Methods of producing ribozymes and gene therapy utilizing these ribozymes also are provided.

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RIBOZYME THERAPY FOR RESTENOSIS

BACKGROUND OF THE INVENTION

In 1992, an excess of 300,000 angioplasties were performed in the United States. Restenosis is a major complication following angioplasty, occurring in 30-60% of patients. Indeed, restenosis is the single most significant problem in interventional cardiology and costs the health care system in excess of $ 1 billion per year.

Restenosis following angioplasty is the result of local vascular injury characterized by local infiltration of platelets, macrophages and local activation of the clotting system. These factors result in the elaboration of the number of biologic mediators of smooth muscle cell (SMC) migration and proliferation. Medial SMCs migrate into the vascular intima and begin to proliferate and produce extracellular matrix (ECM), resulting in the formation of a fibrocellular mass which can obstruct blood flow.

The role of growth factors and cytokines as mediators of SMC migration and proliferation has gained widespread attention. Further, injury has been shown to induce the expression of a variety of oncogenes that are believed to play a role in the cellular response to this injury. Thus, a need exists for an effective therapy to prevent and treat restenosis. This invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

As an effective therapy for restenosis, this invention provides ribozymes and ribozyme delivery systems useful to inhibit abnormal smooth muscle cell proliferation in vascular tissue. Methods of producing ribozymes and gene therapy utilizing these ribozymes also are provided.

DETAILED DESCRIPTION OF THE INVENTION

Restenosis is a major clinical problem and as the result of a need for repeat hospitalization, repeat angioplasty or bypass surgery, restenosis costs the nation's health care system in excess of $1 billion per year. Restenosis is now understood to comprise three important components. First, myointimal proliferation of vascular smooth muscle cells and the subsequent deposition of ECM results in a fibrocellular mass which can encroach upon the vascular lumen. Second, following acute angioplasty, there may be significant elastic recoil of the artery which contributes to a late loss of luminal dimension. Finally, platelets and thrombus adherent to the vascular wall may, over time, organize into a fibrocellular mass.

Smooth muscle cells (SMCs) have been demonstrated capable of producing and responding to a variety of growth factors including platelet-derived growth factor (PDGF), transforming growth factor (TGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), and interleukins. These same factors have been found in human restenotic lesions. In addition, a variety of oncogenes (e.g., c-myc, c-fos, and c-myb) have been found to be involved in smooth muscle cell migration and proliferation as well as deposition of ECM that is associated with post-vascular injury. Smooth muscle cells themselves are capable of regulating their own growth by local autocrine and paracrine mechanisms.

Therefore, by interfering with the local production and action of the growth factors, oncogenes and cell regulatory proteins involved with SMC growth following vascular injury, one can effectively treat and prevent restenosis. This invention provides such by providing ribozymes and methods of using ribozymes that directly block the production of the growth factors, oncogenes and cell regulatory proteins involved with SMC growth following vascular injury.

As used herein, "ribozymes" are intended to include RNA molecules that contain anti-sense sequences for specific recognition, and a RNA-cleaving enzymatic activity. The catalytic strand cleaves a specific site in a target RNA at greater than stoichiometric concentration. Two "types" of ribozymes are particularly useful in this invention, the hammerhead ribozyme (Rossi, J.J. et al., Pharmac. Ther. 50:245-254 (1991) incorporated herein by reference) and the hairpin ribozyme. (Hampel et al., Nucl. Acids Res. 18:299-304 (1990) and U.S. Patent No. 5,254,678, issued October 19, 1993, each incorporated herein by reference.) Because both hammerhead and hairpin ribozymes are catalytic molecules having antisense and endoribonucleotidase activity; ribozyme technology has emerged as a potentially powerful extension of the antisense approach to gene inactivation.

Accordingly, this invention provides a ribozyme having the ability to inhibit pathologic smooth muscle proliferation and restenosis. This ribozyme can be a hammerhead (for example, as described by Forster and Symons (1987) Cell 48:211-220; Haseloff and Gerlach (1988) Nature 328:596-600; Walbot and Bruening (1988) Nature 334:196; Haseloff and Gerlach (1988) Nature 334:585, each incorporated herein by reference) or a hairpin ribozyme (for example, as described by Haseloff et al., U.S. Patent No. 5,254,678, issued October 19, 1993 and Hempel et al.,

European Patent Publication No. 0 360 257, published March 26, 1990, each incorporated herein by reference) having the ability to specifically target, cleave and inactivate factors responsible for restinosis. In one embodiment, the ribozyme has the ability to inhibit the activity of a growth factor responsible for abnormal smooth muscle cell proliferation in vascular tissue. Such growth factors include, but are not limited to platelet derived growth factor, fibroblast growth factor, insulin-like growth factor, c-myc, c-myb, c-fos, cdc2 kinase, TGF-α, TGF-ß, interleukin, and components of the extracellular matrix. As used herein, the term "abnormal smooth muscle cell proliferation" shall mean any small cell proliferation and deposition of extracellular matrix occurring in response to vascular injury or trauma involved by any angioplasty, stent, balloon angioplasty, atherectomy, laser surgery, endovascular or surgical procedure.

The sequence requirement for the hairpin ribozyme is any RNA sequence consisting of NNNG/CN*GUCNNNNNNNN (where N*G is the cleavage site, and where N is any of G, U, C, or A). The sequence requirement at the cleavage site for the hammerhead ribozyme is any RNA sequence consisting of NUX (where N is any of G, U, C, or A and X represents C, U or A) can be targeted. Accordingly, the same target within the hairpin leader sequence, GUC, is useful for the hammerhead ribozyme. The additional nucleotides of the hammerhead ribozyme or hairpin ribozyme is determined by the target flanking nucleotides and the hammerhead consensus sequence. This information, and the published sequence of nucleic acids coding for c-myc, c-myb, c-fos, cdc2 kinase, TGF-α and TGF-ß proteins enables the production of the ribozymes of this invention. Appropriate base changes in the ribozyme is made to maintain the necessary base pairing with the target RNA sequences.

Cech et al. (U.S. Patent No. 4,987,071, issued January 22, 1991) has disclosed the preparation and use of certain synthetic ribozymes which have endoribonuclease activity. These ribozymes are based on the properties of the Tetrahymena ribosomal RNA self-splicing reaction and require an eight base pair target site. A temperature optimum of 50°C is reported for the endoribonuclease activity. The fragments that arise from cleavage contain 5′ phosphate and 3′ hydroxyl groups and a free guanosine nucleotide added to the 5′ end of the cleaved RNA. In contrast, the ribozymes of this invention hybridize efficiently to target sequences at physiological temperatures, making them suitable for use in vivo, not merely as research tools (see, column 15, lines 18-42, of Cech et al., U.S. Patent No. 4,987,071).

The ribozymes of this invention and DNA encoding the ribozymes, described in more detail below, can be chemically synthesized using methods well known in the art. (For example, according to recommended protocols of Promega, Madison, Wis., USA, incorporated herein by reference). The ribozymes also can be prepared from a DNA molecule (that upon transcription, yields an RNA molecule) operably linked to an RNA polymerase promoter, e.g., the promoter for T7 RNA polymerase or SP6 RNA polymerase. Accordingly, also provided by this invention is a nucleic acid molecule, i.e., DNA or cDNA, coding for the ribozymes of this invention. When the vector also contains an RNA polymerase promoter operably linked to the DNA molecule, the ribozyme can be produced in vitro upon incubation with RNA polymerase and nucleotides. In a separate embodiment, the DNA is inserted into an expression cassette as described in Cotten and Birnstiel (1989) EMBO J. 8(12):3861-3866 and in Hempel et al., Biochemistry 28:4929-4933 (1989), each incorporated herein by reference. A more detailed discussion of molecular biology methodology is disclosed in Sambrook et al. (1989) Molecular Cloning: A

Laboratory Manual, Cold Spring Harbor Press, incorporated herein by reference. After synthesis, the RNA molecule can be modified by ligation to a DNA molecule having the ability to stabilize the ribozyme and make it resistant to RNase. Alternatively, the ribozyme can be modified to the phosphothio analog for use in liposome delivery systems. This modification also renders the ribozyme resistant to endonuclease activity.

The DNA molecule also can be in a host procaryotic or eucaryotic cell in culture or in the cells of an organism. Appropriate procaryotic and eucaryotic cells can be transfected with an appropriate transfer vector containing the DNA molecule encoding a ribozyme of this invention. When the DNA molecule is operatively linked to a promoter for RNA transcription, the RNA can be produced in the host cell when the host cell is grown under suitable conditions favoring transcription of the DNA molecule. The vector can be, but is not limited to a plasmid, a virus, a retrotransposon or a cosmid. Examples of such vectors are disclosed in U.S. Patent No. 5,166,320, incorporated herein by reference. A suitable adenoviral vector such as adeno-associated vector type 1 ("AAV-1") or adeno-associated vector type 2 ("AAV-2") (see Chatterjee et al., (1992) Science Vol. 258:1485-1488, incorporated herein by reference) are particularly useful. Methods of gene therapy are well known in the art, see, for example, Larrick, J.W. and Burck, K.L. Gene Therapy: Application of Molecular Biology, Elsevier Science Publishing Co., Inc. New York, New York (1991) and Kreigler, M. Gene Transfer and Expression: A Laboratory Manual, W.H. Freeman and Company, New York (1990), each incorporated herein by reference.

To produce the ribozymes with a vector, the nucleotide sequences coding for ribozymes are placed under the control of a strong promoter such as the lac, SV40

late, SV40 early, or lambda promoters. Ribozymes are then produced directly from the transfer vector in vivo.

In a separate embodiment, the viral vector is a retrovirus, such as a non-virulent vaccinia based viral vector, a Moloney murine leukemia virus, or an HIV vector as described in Lever, A. (1989) J. Virol. 63:4085-4087, incorporated herein by reference. Retroviral vectors are particularly useful for gene therapy of restinosis. An appropriate retroviral vector for such gene therapy is a self-replicating retrovirus having inserted between the 5′ and 3′ long terminal repeat (LTR) regions of the retrovirus a foreign nucleic acid (DNA or cDNA) sequence, under the control of a promoter, for example, the retroviral LTR or an inserted pol III promoter such as the human $tRNA^{Val}$ promoter or the adenovirus VA1 promoter. The retroviral vector then stably expresses the ribozyme in the host cell.

Further provided by this invention are retroviral vectors having inserted between the 5′ and 3′ LTR more than one nucleic acid molecule encoding a ribozyme of this invention, each molecule under the control of a separate pol III promoter or alternatively, under the control of single pol III promoter or the retroviral LTR. These retroviral vectors provide the advantage of providing multi-functional therapy against restenosis, each specific therapy working in synergy. In addition, because only a limited number of vectors are used, the number of integration sites in the host cell are reduced thereby reducing the possibility of activation of host cellular DNA sequences by the inserted retroviral vector.

Host procaryotic and eucaryotic cells stably transduced with the vectors described above also are provided by this invention. Suitable host cells include

bacterial cells, mouse cells, rat cells, mouse cells, and human cells, for example, vascular smooth muscle cells.

A method of inhibiting abnormal smooth cell vascular tissue proliferation or restenosis is provided by this invention. This method requires contacting a suitable cell or tissue with an effective amount ribozyme of this invention or alternatively, by transducing the cell with an effective amount vector having a nucleic acid molecule encoding the ribozyme. Effective amounts are easily determined by those of skill in the art using well known methodology. When exogenously delivering the ribozyme, the RNA molecule can be embedded within a stable RNA molecule or in another form of protective environment, such as a liposome. Alternatively, the RNA can be embedded within RNase resistant DNA counterparts. Cellular uptake of the exogenous ribozyme can be enhanced by attaching chemical groups to the DNA ends, such as cholesteryl moieties (Letsinger et al., P.N.A.S., U.S.A. (1989), incorporated herein by reference).

In a separate embodiment, the target cell is transduced under conditions favoring insertion of the vector into the target cell and stable expression of the nucleic acid encoding the specific ribozyme. The target cell can include but is not limited to a vascular smooth muscle cell or a cell responsible for the deposition of proteins involved in the formation of extracellular matrix. Such cells are well known to those of skill in the art. Accordingly, another aspect of this invention is a method for interfering with or preventing abnormal smooth muscle proliferation in a suitable cell by reacting the target RNA sequence with a ribozyme of this invention. Within the cell or within the cells of an organism, a transfer vector as described above encoding one or more ribozymes is transfected into a cell or cells using methods described in Llwewllyn et al., (1987) J. Mol. Biol. 195:115-123 and

Hanahan et al. (1983) 166:557-580, each incorporated herein by reference. Inside the cell, the transfer vector replicates and the DNA coding for the ribozyme is transcribed by cellular polymerases to produce ribozymes which then inactivate factors responsible for abnormal smooth muscle cell proliferation, abnormal extracellular matrix deposition and restinosis. Micromanipulation techniques such as microinjection also can be used to insert the vector into the cell so that the transfer vector or a part thereof is integrated into the genome of the cell. Transcription of the integrated material gives rise to ribozymes which then inactivate the target proteins. As used herein, the term "inactivate" is intended to mean interfere with the production of the protein product such as c-myc or TGF-ß.

Compositions also are provided by this invention. These compositions contain any of the above described ribozymes, DNA molecules, vectors or host cells. In one aspect of this invention, the compositions also contain a pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents.

Host cell containing the ribozymes, nucleic acids and/or vectors described above also are within the scope of this invention. These host cells can be procaryotic cells, for example bacterial cells or eucaryotic cells, such as mammalian, human, rat, or mouse cells. The host cells transduced with nucleic acids encoding the ribozmyes are useful to recombinantly produce the ribozymes. Thus, also provided by this invention is a method for producing a ribozyme, in vitro or in vivo, the ribozyme being able to inhibit abnormal smooth muscle cell proliferation in

vascular tissue. DNA encoding the ribozyme is provided to the cell, the DNA being under the transcriptional control of a promoter, using methods well known to those of skill in the art. See Sambrook et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1989), incorporated herein by reference.. The DNA is then transcribed in the cell to produce the ribozyme. When produced in vitro, the ribozyme can be purified or isolated from the cell by using methods well known in the art. The DNA can be transferred in a carrier or in a vector in a carrier a number of ways. For example, the DNA can be administered by transluminal delivery to the vascular wall, or exoluminally. In another aspect, the active ingredient can be embedded in a biodegradable polymer or sphere and administered by vascular stent. Alternatively, it can be delivered in a pleuronic gel.

Further provided by this invention are methods of inhibiting abnormal smooth muscle cell proliferation in vascular tissue, by introducing into the cell one or more of the ribozymes described above, in an amount effective to inhibit SMC proliferation or abnormal extracellular matrix deposition.

An alternative method of inhibiting abnormal smooth muscle cell proliferation in vascular tissue consists of introducing into a cell an effective amount of DNA encoding a ribozyme described above, under conditions favoring transcription of the DNA to produce the ribozyme. This method also is useful to prevent abnormal SMC and abnormal extracellular matrix deposition and thus, prevent restenosis.

Also provided by this invention is a method of inhibiting or preventing abnormal smooth muscle cell proliferation in vascular tissue in a subject, which comprises administering to the subject an effective amount

of a ribozyme having the ability to inhibit abnormal smooth muscle cell proliferation in vascular tissue. The ribozyme is delivered to the smooth muscle cell exoluminally, transluminally, by stent, by a biodegradable polymer or sphere or in a pleuronic gel.

It should be understood to those skilled in the art that "effective amounts" are administered. These amounts are easily determined using methods well-known to those of skill in the art.

Although the invention has been described with reference to the presently preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A ribozyme having the ability to inhibit abnormal smooth muscle cell proliferation in vascular tissue.

2. The ribozyme of claim 1, wherein the ribozyme has the ability to inhibit the activity of a growth factor responsible for abnormal smooth muscle cell proliferation in vascular tissue.

3. The ribozyme of claim 2, wherein the growth factor is platelet derived growth factor, fibroblast growth factor, TGF-α, TGF-ß, c-myc, c-fos, c-myb, cdc2-kinase, insulin-like growth factor or an interleukin.

4. The ribozyme of claim 2, wherein the ribozyme specifically cleaves a protein responsible for the production of an extracellular matrix protein.

5. The ribozyme of claim 1, wherein the ribozyme has the ability to prevent abnormal deposition of extracellular matrix in vascular tissue.

6. A nucleic acid molecule encoding the ribozyme of claim 1.

7. The nucleic acid molecule of claim 6, wherein the nucleic acid is DNA or cDNA.

8. The nucleic acid molecule of claim 6, under the control of a promoter to transcribe the nucleic acid.

9. A host cell comprising the ribozyme of claim 1.

10. A vector comprising the nucleic acid of claim 6.

11. A host cell comprising the vector of claim 10.

12. The vector of claim 10, wherein the vector is a plasmid, a virus, retrotransposon or a cosmid.

13. The vector of claim 12, wherein the vector is an adenoviral vector or an AAV.

14. A host cell stably transformed with the vector of claim 13.

15. The host cell of claim 9 or 14, wherein the host cell is a human cell.

16. A method for producing a ribozyme, the ribozyme being able to inhibit abnormal smooth muscle cell proliferation in vascular tissue, which comprises providing DNA encoding the ribozyme under the transcriptional control of a promoter, transcribing the DNA to produce the ribozyme.

17. The method of claim 16, wherein the ribozyme is produced in vitro.

18. The method of claim 17, further comprising purifying the ribozyme produced.

19. The method of claim 16, wherein the ribozyme is produced in vivo.

20. A method of inhibiting abnormal smooth muscle cell proliferation in vascular tissue, which comprises introducing into the cell an effective amount of the ribozyme of claim 1.

21. A method of inhibiting abnormal smooth muscle cell proliferation in vascular tissue, which comprises introducing into the cell an effective amount of the DNA of claim 6 under conditions favoring transcription of the DNA to produce the ribozyme.

22. The method of claim 20 or 21, wherein the cell is a human cell.

23. A method of preventing abnormal smooth muscle cell proliferation in vascular tissue, which comprises introducing into the cell an effective amount of the DNA of claim 6 under conditions favoring transcription of the DNA to produce the ribozyme.

24. The method of claim 23, wherein the cell is a human cell.

25. A method of inhibiting or preventing abnormal smooth muscle cell proliferation in vascular tissue in a subject, which comprises administering to the subject an effective amount of the ribozyme of claim 1.

26. The method of claim 25, wherein the ribozyme is delivered to the smooth muscle cell exoluminally, transluminally, by stent, by a biodegradable polymer or sphere or in a pleuronic gel.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/70; C12N 5/16, 9/22, 15/85, 15/86; C12Q 1/25

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.21, 91.3, 91.31, 172.3, 240.2, 320.1; 536/23.2, 24.5; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG: BIOSIS, MEDLINE, Derwent Biotechnology Abstracts, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. Cell. Biochem. Suppl., Vol. 17 Part B, issued 1993, Content et al., "Construction of a ribozyme cleaving human IL-6 mRNA", page 110, Abstract No. E 502, see entire abstract.	1-26
X	Proc. Am. Assoc. Cancer Res. Annu. Meet., Volume 31, issued March 1990, Scanlon et al., "Development of a c-*fos* ribozyme to circumvent cisplatin resistance in human ovarian carcinoma cells", page 404, Abstract No. 2399, see entire abstract.	1-26
X	Journal of Molecular Biology, Vol. 242, No. 5, issued 1994, Sioud, "Interaction between tumour necrosis factor alpha ribozyme and cellular proteins. Involvment in Ribozyme Stability and Activity", pages 619-629, see entire document.	1-26

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"O"	document referring to an oral disclosure, use, exhibition or other means		
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Proc. Natl. Acad. Sci. USA, Vol. 88, issued December 1991, Scanlon et al., "Ribozyme-mediated cleavage of c-fos mRNA reduces gene expression of DNA synthesis enzymes and metallothionein", pages 10591-10595, see entire document.	1-26
X	Advan. Enzyme Regul. Volume 32, issued 1992, Funato et al., "The utility of an anti*fos* ribozyme in reversing cisplatin resistance in human carcinomas", pages 195-209, see entire document.	1-26
X	Journal Interferon Research, Volume 12, Supplement 1, issued September 1992, Mahieu et al., "Construction of a ribozyme cleaving human IL-6 mRNA", page S79, Abstract No. P4-6, see entire abstract.	1-26
X	WO, A, 91/02595 (SULLIVAN ET AL.) 03 February 1994, see entire document.	1-26
X	WO, A, 92/17206 (FERGUSON ET AL.) 15 October 1992, see entire document.	1-26
X	WO, A, 91/15580 (FUNG ET AL.) 17 October 1991, see entire document.	1-26
X	WO, A, 93/23057 (THOMPSON ET AL.) 25 November 1993, see entire document.	1-26
X	WO, A, 93/20691 (TYKOCINSKI ET AL.) 28 October 1993, see entire document.	1-26
X	WO, A, 91/18012 (CLARK ET AL.) 28 November 1991, see entire document.	1-26
X	WO, A, 92/00990 (BURCH) 23 January 1992, see entire document.	1-26

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER:
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435/6, 91.1, 91.21, 91.3, 91.31, 172.3, 240.2, 320.1; 536/23.2, 24.5; 514/44