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Seæor
SUPERINTENDENTE DE INDUSTRIA Y COME
DIVISION DE NUEVAS CREACIONES

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iIIIIiIJIOIJIflhIIilhIf$IJliJIgIH No. 06-013004.. -00004-0000
t 162342 i)D 2,020 ’11 PAILNIEJY?j
At L t HA L’ 44-1, 1ci:o: 1

Re: P1.-WYETH.-Solicitud de Patente de Invención en Fase Naciorial PCT
parp ’MOLECULAS DE LUBRICINA RECOMBINANTES Y USOS DE LAS MISMAS".-Clase A61K 38/17: 31/728; 39/395; A61P 19/02; C07K 14/435.-Expediente nimero 06-013.004..-

1. Me refiero al auto dictado por ese Despacho notificado por fijación en lista de
12 de Marzo de 2010.

2. Con elfin de responder las objeciones presentadas por el Seæor Examinador en
su Concepto TØcnico No. 690 bajo los acÆpites de Claridad del CapItulo reivindicatorio, Unidad de Invención, No es una invenaón y Excepción a la patentabilidad, me permito dirigir su atención a las siguientes modificaciories y argumentos tØcnicos, por medio de los cuales se superan completamente dichas
objeciones.

3. CAPITULO REIVINDICATORIQ.

En primera instancia, el Seæor Examinador objeta la claridad del capItulo
reivindicatorio actualmente preseritado por considerar que este no muestra las caracterIsticas que son compartidas por la materia reclamada. AsI mismo, el Seæor
. Examinador seæala que es necesario precisar el origen de las protelnas reivindicadas para excluir la posibilidad de que estas corresporiden a molØculas existentes en la
naturaleza.

Tomando en consideración estas objeciones, es deseo del solicitante modificar
el capitulo reivindicatorio actualmente en estudio de forma que sea atin mØs claro el
objeto de la presente invención.

Con respecto a estas modificaciones, me permito resaltar que no coristituyeri de ninguna manera una ampliación al alcance de la invención, tal como fue originalmente presentada, toda vez que se estÆn incluyendo caracterIsticas particulares de la invención reveladas dentro del texto de la descripción, las cuales hacen aUn mths clara la materia reclamada y ayudan a distinguirla del arte previo.

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De esta manera dentro de las reivindicaciones 1, 2, 5 y 11 se ha introducido Ia aclaración con respecto a que Ia proteina aislada reclamada es una proteIna recombinante, tal como se manifiesta de forma clara a lo largo de todo el texto de la descripción:

"La presente invención se refiere a nuevas molØculas de lubricina recombinantes y a su usa coma lubricantes, agentes antiadhesivos yb suplementos intra-articulares." (Pgina 3, lIneas 11 a 13, descripción de la solicitud en estudio, resaltado fuera de texto).

En relación con lo anterior me permito seialar que dentro del contexto de la presente invención el tØrmino "aislado" no implica una separación del medio de una molØcula existente en la naturaleza, sino que hace referencia al grado de pureza de la molØcula de interØs, tal como se define dentro de la pÆgiria 16 dela descripción de Ia solicitud en estudio:

"Los po/ipØptidos -otros compuestos descritos en la presente se dice que son "aislados" cuando estØn dentro de preparaciones que son a! menos 50% en peso (peso seco) el cornpuesto de interØs. Los polipØptidos u otros cornpuestos descritos en la presente se dice que son "sustancialmente puros" cuando estÆn dentro de preparaciones que son a! menos 80% en peso (peso seco) el cornpuesto de interØs. - (PÆgina 16, lIneas 1 a 7, descripción de Ia solicitud en estudio, resaltado fuera de texto).

Como consecuencia de lo anterior, al referirse el capitulo reivindicatorio a una "proteIna a/s/ada" no se estÆ hacienda alusión a una molØcula que existe dentro de la naturaleza y que es identificada y purificada, sino que se pretende reclamar una molØcula la cual se encuentra en una preparación con a! menos 50% en peso seco del compuesto de interØs.

Este hecho es tambiØn evidente si se observan las secuencias incluidas dentro
del capItulo reivindicatorio, las cuales difieren de forma significativa de aquellas correspondiente a proteInas existentes en la naturaleza y si se observa el ya mencionado texto de la descripción de la solicitud en estudio que resalta que todas las proteInas objeto de la invención son recombinantes, muestra los constructos a partir de las cuales se obtienen dichas protelnas y los vectores y mØtodos por los cuales se expresan dichos constructos.

Como consecuencia de lo anterior, la modificación realizada sobre las mencionadas reivindicaciones 1, 2, 5 y 11 hace totalmente claro el hecho que la presente invención se refiere a protelnas recombinantes y no a ninguna sustancia extraIda como tal de la naturaleza.

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Ahora bien, con respecto a las caracteristicas tØcnicas que comparte la materia reclamada me permito resaltar que todas las proteInas que son objeto de la presente invención comparten la secuencia KEPAPTT y tienen una actividad lubricante. Adems, todas estas protelnas poseen una drÆstica reducción en las unidades repetitivas de KEPAPTT comparadas con la protelna nativa y, gracias a esta modificacióri, las protelnas de la invención retienen la actividad lubricante y son relativamente sencillas de sintetizar y manipular para realizar por ejemplo producción bioterapØutica a gran escala.

De esta forma, todas las protelnas reclamadas por la presente solicitud poseen tres caracterIsticas principales: estn relacionadas en su secuencia con la lubricina, poseen unidades repetitivas KEPAPTT y tienen actividad lubricante. Esto es claro a partir de Ia descripción de la solicitud en estudio, por ejemplo la tabla 2 de las pÆginas26 y 27 de dicha descripción:

LUB:1 19
APTTPP.PPTTTKSAPTTPKEPAPTTTKEPAPTTPKEPAPTTTKpApT

LUB:2 113
APTTPKEPAPTTTKSAPTTPKEPPTTTKEPAPTTFKEPAPTTTKEPAPTTTK SAPTTPPAPTTPKEPKPAPTTP

LUB:3 117
APTTPPAPTTTKSAPTTPKPAPTTTKEPAPTTPKEPAPTTTKPA1TTTK SAPTTPICEPARTTPKEPAPTTTEEPAPTTTKSAPTTPIKEPAPTTPKEPKPAPT TP
LUB:4 121
APTTPKEPAPTTTKSAPTTPKEPAPTTTEEPAPTTPKEPAPTTTREPAPTTTK SAPTTPKEPAPTTPKEPAPTTTPAPTTTKSAPTTPI’EPAPTTPKEPAPTTT KEPAPTTTKSAPTTPKEPATTPKEI?KPAPTTP

LUB:5 1 25
APTTPPAPTTTKSAPTTPKPAPTTTKEPAPTTPcPAPTTTBEpAPTTTK SAPTTPKEPPTTPKEPAPTTTKEPAPTTTKSAPTTPKEPAPTTPKEP1TT KEPAPTTTKSAPTTPKEPAPTTPKEPAPTTTKEAPTTTKSAPTTP?ATT ?K1PKPAPTTP

De igual manera, las secuencias SEQ ID NO: 26 y 27 poseen estas mismas caracterIsticas:

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"En esta modal/dad preferida, la secuencia de aminoÆcidos "APJTPKEPAPTTTKSAP7TPKEPAPTTTKEPAPTTPKEPAPTTTK" (SEQID NO: 26; 45 aminoÆcidos) permanece comoparte de cada proteIna PRG4-LU6:N (en donde N = un entero de 1 o mÆs). AdemÆs, la secuencia de aminoÆcidos "KEPAPTTTKEPAPTTTKSAP7TPKEPAP17P" (SEQ ID NO: 27; 31 aminoÆcidos) es codificada por el inserto de ADN que se vuelve parte de cada construcciÆnde ADNc PRG4-Lub:N+1 a travØs de la adición del casete-2 de ADNc sintØtico 8su361/BspEI a una construcción de ADNc PRG4-Lub:N." (Pgina 30, lIneas 1 a 10, descripción de la solicitud en estudio).

Asi mismo, las protelnas completas PRG4:LUB: 1, 2, 3, 4 y 5 (SEQ ID NO: 7,
11, 15, 19 y 23) dentro de su secuencia poseen la unidad KEPAPTT (Lys-Glu-Pro-Ala-Pro-Thr-Thr) varias veces, por ejemplo la SEQ ID NO: 23 de la pgina 36 del listado
de secuencias posee esta uriidad repetitiva en los residuos 394-400, 402-409, 418-

442, 433-439, 449-455, 471-478, 480-488 y 511-517. IR

De esta forma, todas las proteInas reclamadas por la presente solicitud
comparten las mismas caracteristicas tØcnicas, que serlan evidentes para la persona versada en la materia a partir de sus secuencias y de lo revelado dentro de la descripción de la solicitud en estudio.

En relación con lo anterior, me permito seæalar que las secuencias de
nucleótidos reclamadas por el capItulo reivindicatorio son aquellas que codifican a estas prot&nas particulares de la invención y por lo tanto tambiØn comparten caracteristicas tØcnicas con dichas protelnas. AsI mismo, el mØtodo de obtener las proteInas de la invención y el vector empleado en este mØtodo estØn Intimamente relacionados con dichas protelnas.

De manera similar, las composiciones a las cuales se hace referenda dentro del capItulo reivindicatorio contienen las proteInas objeto de la invendión y por lo tanto estÆntØcnicamente relacionadas entre si.

En vista de lo anterior, es evidente que el capItulo reivindicatorio es claro al reclamar el objeto de Ia presente invención y que toda Ia materia alil considerada comparte las caracteristicas tØcnicas principales reveladas dentro de la descripción de Ia solicitud en estudio.

De otra parte, me permito seæalar que es deseo del solicitante eliminar las reivindicaciones 22 a 27 actualmente presentadas.

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Sumado a lo anterior, el solicitante desea incluir las nuevas reivindicaciones
27 a 33 las cuales se refieren a caracterIsticas distintivas de las protemnas recombinantes, las composiciones y el mØtodo objeto de la presente invención, caracteristicas que estÆn contenidas en las pÆginas 34, 41, 43, 44, 48 y 49 de la descripción de la solicitud en estudio.

En relación con las nuevas reivindicaciones 27 a 33, me permito seæalar que estas no pretenden de ninguna manera ampliar el alcance de la invenciórl, tal como fue originalmente presentada, toda vez que dichas reivindicaciones estÆn haciendo referencia a caracterIsticas particulares que permiten distinguir la invención del arte previo, Jas cuales ya hablan sido reveladas dentro de la descripción de la solicitud.

Como consecuencia de las modificaciones anteriores el capItulo reiviridicatorio modificado queda constituido par 33 reivindicaciones que corresponden a las reivindicaciones 1 a 21 y 28 a 32 ariteriormente presentadas renumeradas como nuevas reivindicaciones 1 a 26, junto con las nuevas reivindicaciones 27 a 33 que defirien mÆs detalladamentela materia reclamada por la presente invención.

Adicionalmente, dentro de las reivindicaciones 2, 3, 4 y 5 se ha restringido el valor de "N" de forma que corresponda mximo a 25 de acuerdo a las realizaciones preferidas de la invención y a lo enseæado en la pgina 32 de la descripción de la solicitud en estudio.

Asi mismo, se ha cambiado la dependencia de Pa reivindicación 19 para que haga referencia tambiØn a la proteIna de la reivindicación 11, asI como se ha modificado la dependencia de la reivindicación 32 (nueva reivindicación 26) para que haga alusión Cinicamente a Pa protelna de acuerdo con la reivindicación 11.

Como consecuencias de estas modificaciones, el capitulo reivindicatorio es totalmente claro al reclamar el objeto de Ia presente invención y por lo tanto se ajusta completamente a los requerimientos exigidos par el ArtIculo 30 de la Decision Andina 486 de 2000.

4. UNIDAD DE ZNVENCIÔN.

Por otro lado, el Seæor Examinador seæala que el capItulo reivindicatorio carecerIa de unidad de invención toda vez que seqün su anlisis "contempla diferentes conceptos inventivos’ç seæalando adems con respecto a las protelnas y los polinucleótidos reclamados "entre el/os no existe una relación dado que cada polipØptido es diferente de los otros y cada p0/inuc/eôtido es diferente de los otros polinucleótidos. Por /0 tanto, las reivindicaciones que se relacionan con los

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polipØptidos no cornparten la misma caracteristica tØcnica esencial ni las que se re/acionan con los polinucleÆtidos. Cada secuencia es estructural, qulmica y funcionalmente diferente de las otras".

Sobre este particular me permito seæalar que, tal como se discutió en el numeral anterior de este mismo memorial, toda la materia reclamada por la presente solicitud estÆ relacionada por las mismas caracteristicas tØcnicas y por Ia tanto, comparte el mismo concepto inventivo y se constituye en una sola unidad de invención.

De esta forma, todas las proteInas objeto de la presente invención son proteInas recombinantes que poseen actividad lubricante y que tienen dentro de su secuencia unidades repetitivas KEPAPTT. Este hecho es claro a partir de las secuencias de dichas protelnas, como se puede revela en la tabla 2 de las pginas 26 y 27 de la descripción de la solicitud, asI como en las pÆginas 30 a 32 de dicha descripción y en el listado de secuencias anexo a la solicitud.

Coma consecuencia de lo anterior, todas las protelnas reclamadas por la presente solicitud estÆn estructuralmente y funcionalmente relacionadas, toda vez que todas ellas tienen regiones repetitivas comunes en su secuencia y su actividad biológica es la misma.

De otra parte, me permito seæalar que, contrario a lo afirmado por el Seæor Examinador, el hecho que dos secuencias sean distintas entre si no necesariamente implica que no compartan el mismo concepto inventivo.

En este sentido me permito resaltar que, dentro del Æmbitode las patentes, es ampliamente aceptado y conocido que la materia revelada en una invención podr coexistir en una sola solicitud si existe una relación tØcnica entre las posibles realizaciones que involucre una o ms de las mismas caracterIsticas tØcnicas especiales (Regla 13.1 del PCT). Dichas "caracterIsticas tØcnicas especiales" son definidas por Ia Regla 13.2 del Tratado de Cooperación en materia de Patentes (PCT)

Como:

"La expresión "caracterIsticas tØcnicas especiales" significarÆ aquellas caracterIsticas tØcnicas que definen una contribuciÆn quecada una de las invenciones reclamadas, consideradas como un todo, hace sobre el arte previo".

Es decir, si toda la materia que estÆ incluida dentro del capItulo reivindicatorio
de una solicitud comparte caracterIsticas tØcnicas que son las que distinguen y constituyen el aporte hecho por la solicitud al campo tØcnico de la invención, se considerar6 que dicha solicitud posee unidad de invención.

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Ahora bien Para el caso aue nos cpmDete todas las DroteInas ciue son reclampdps oor la presente invenciôn estÆn relacionadas estructuralmente con IM lubricina, tienen dentro de su estructura unidades reDetitivas de KEPAPTT (aunciue mucho menos unidades pue la corresDondiente lubricina nativa)

poseen actividad lubricante v antiadherente, siendo todas estas las carpcterIsticas aue distinciuen la invención del camno tØcnico v ciue constituven su aDorte sobre

arte Drevio

Adicionalmente, como se discutió en el numeral anterior del presente escrito, al tener menor cantidad de unidades KEPAPTT todas las protelnas de la inverición resultan mÆs fÆcilesde sintetizar y manipular, por ejemplo en producción a gran escala pero retienen las propiedades lubricantes y antiadherentes deseadas, to que constituye una gran contribución al campo tØcnico de la invención.

De esta forma, todas proteInas que hacen parte del capItulo reivindicatorlo de
la solicitud en estudio comparte el mismo concepto inventivo.

Por su parte, los polinucleótidos considerados dentro de dicho capItulo reivindicatorio son aquellos que codifican las protelnas de la invención y por Ic tanto estÆn tØcnicamente relacionadosbajo el mismo concepto inventivo.

De manera similar, el mØtodo para obtener las protelnas recombinantes de la
invención y el vector de expresión empleado en dicho proceso tienen relación tØcnica
estrecha con las mencionadas proteInas de la invención y por lo tanto comparten el
mismo concepto inventivo.

AsI mismo, las composiciones farmacØuticas que hacen parte de la materia Sreclamada por la invención comprenden las protelnas recombinantes desarrolladas por el solicitante, y por lo tanto comparten las mismas caracterIsticas tØcnicas
especiales exhibidas por dichas protemnas.

En vista de todo Ic anterior es dare que la solicitud en estudio reclama materia
que comparte el mismo concepto inventivo y por to tanto cumple a cabalidad con lo preceptuado por el ArtIculo 25 de la Decision Andina 486 de 2000.

5. NO ES UNA INVENCIÔN.

Adicionalmente, en el referenciado Concepto TØcnico No. 690 el Seæor Examinador indica que las reivindicaciones 11 y 12 hacen referenda a protemnas que no serIan una invención, toda vez que segün su anÆlisisestas son aislodas a partir de la naturaleza ya que comprenden las secuencias completas.

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Sobre este particular me permito seæalar que las mencionadas reivindicaciones se refieren a las proteInas con SEQ ID Nos: 7, 11, 15, 19 y 23, las cuales aun cuando son proteInas completas no corresponden a ninguna proteina nativa, sino que son protelnas recombinantes.

Esto es claro a partir de las eriseæanzas de la descripción de la solicitud en estudlo:

"La construcción de ADNc PRG4-Lub:1 recombinante (SEQ ID NO: 6) en
el vector pTmed2 (construcciôn mÆs vectorigual a SEQ ID NO: 5) es flanqueada por
SitiOS de restricción Sail (G"TCGAC; nos. de base 1027 a 1032 de SEQ ID NO: 5) y
Non (GC"GGCCGC; nos. de base 3984 a 3991 de SEQ ID NO: 5). (...)

La construcción de ADNc PRG4-Lub:1 (SEQ ID NO: 6) se traduce en la
proteIna PRG4-LUB:1 (SEQ ID NO: 7). El inserto entre 5373 y E425 (es decir,

E848 de PRG4 nativo) de ía proteIna PRG4-LUB:1 completa (SEQ ID NO: 7) es los 51 ID aminoÆcidosde SEQ ID NO: 9. (...)" (PÆgina 22, descripción de la solicitud, resaltado
fuera de texto)

"La construcción PRG4-Lub:2 (SEQ ID NO: 10) se traduce en la protelna PRG4-LUB:2 (SEQ ID NO: 11)." (Pgina 24, descripción de la solicitud, resaltado
fuera de texto).

AsI, las pÆginas 22 a 26 de la descripción de la solicitud en estudio revelan la constitución de los constructos a partir de los cuales son obtenidas las protemnas de SEQ ID Nos: 7, 11, 15, 19 y 23 a las cuales hacen referencia las mencionadas reivindicaciones 11 y 12.

De manera similar, un anlisis detallado del listado de secuencias muestra que dentro de la secuencia de nucleótidos que en cada caso codifica la protelna correspondiente (SEQ ID Nos: 6, 10, 14, 18 y 22) se seæala en el numeral <213> "artificial" y en el numeral <223> "construcción ADNc (..) recombinante". Asi mismo dentro de cada una de las SEQ ID Nos: 7, 11, 15, 19 y 23 en el numeral <213> se seæala"artificial".

Consecuentemente, a pesar del hecho que las mencionadas SEQ ID Nos: 7, 11,
15, 19 y 23 hagan referenda a "proteinas completas" esto no implica que sean idØnticas a protelnas nativas, sino que se estÆ expresando el yen completo y por lo tanto se tienen ms de mil residuos de aminocidos en cada una de ellas.

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Sumado a lo anterior, me permito seæalar que dichas proteInas difiereri de la lubricina nativa por que poseen una deleción importante en la parte central de la proteIna (aproximadamente del 30%), por lo que claramente el capItulo reivindicatorio no hace referenda a ninguna proteIna nativa (la lubricina tiene aproximadamente 1400 residuos en contraste con las protemnas reivindicadas que poseen entre 1000 y 1100 residuos).

Este hecho ha sido clarificado dentro del capItulo reivindicatorio al introducir la expresión recombinante"aI referirse a las mencionadas protemnas.

Adicionalmente, el Seæor Examinador resalta que la reivindicación 32 (nueva reivindicación 26) estarIa dirigida a un anticuerpo natural contra las proteInas de las reivindicaciones 1 y 2 y por lo tanto no serIa considerado como uria irivenciOn.

A este respecto me permito resaltar que como se ha demostrado en este mismo memorial las protelnas reclamadas por Ia presente solicitud no son nativas y por lo tanto, all inducir Ia generación de anticuerpos contra estas proteInas dichos anticuerpos tampoco corresponderÆna anticuerpos nativos.

En relación con lo anterior, me permito seæalar que dentro de las modificaciones realizadas sobre el capItulo reivindicatorio se ha cambiado la dependencia de la reivindicación 32 (nueva reivindicación 26) para que haga
referenda 6nicamente a la reivindicación 11. Es decir que el anticuerpo al que se estÆ
haciendo referencia es creado a partir de las proteInas recombinantes completas de
SEQ ID Nos: 7, 11, 15, 19 y 23, y por lo tanto no corresponde de ninguna martera a
un anticuerpo que pueda existir dentro de la naturaleza.

. Como consecuencia de lo anterior, el capItulo reivindicatorio modificado no se refiere a ninguna molØcula que haya sido obtenida a partir de la naturaleza, y de esta
forma se ajusta totalmente a las exigencias del ArtIculo 15 de Ia Decision Andina
486 de 2000.

6. EXCEPCIÔN A LA PATENTABILIDAD.

Finalmente, el Seæor Examinador seæalaque las reivindicaciones 20 a 27
corresponden a un mØtodo de tratamiento lo cual estÆ exento de pateritabilidad de acuerdo con el ArtIculo 20 de la Decision Andina 486 de 2000.

http://www.ncbLnlmnih.gov/protein/Q92954.2

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Sobre este particular me permito resaltar que, tal como se expuso en el numeral 3 de este mismo memorial las mencionadas reivindicaciones 20 a 27 han sido eliminadas del capItulo reivindicatorio.

De esta forma, el capItulo reivindicatorio modificado no se refiere de ninguna manera a mØtodos de tratamiento y por lo tanto cumple totalmente con lo estipulado en el ArtIcijlo 20 de la Decision Andina 486 de 2000.

7. CONCLUSION.

Por ültimo me permito resaltar que en virtud de las modificaciones realizadas dentro del capItulo reivindicatorio este es totalmente claro y conciso al reclamar el objeto de la presente invención.

De otra parte, los argumentos discutidos en el presente memorial establecieron
de forma incontrovertible que la materia reclamada por el capItulo reivindicatorio modificado estÆ cobijada baja un tnico concepto inventivo, toda vez todas las proteInas reclamadas comparten las mismas caracterIsticas tØcnicas: son protelnas relacionadas con lubricina, con menor nümero de unidades repetitivas KEPAPTT que esta, y que poseen actividad lubricante. De igual forma, los polinucleOtidos que codifican estas protelnas, las composiciones farmacØuticas que las contienen, el mØtodo para obtenerlas y el vector empleado en dicho mØtodo estÆncobijados bajo el mismo concepto inventivo.

Adicionalmente, los mencionados argumentos proporcionados en este escrito demostraron que el capitulo reivindicatorio no se refiere de ninguna manera a molØculas existentes dentro de la naturaleza, sino a molØculas artificiales recombinantes.

Finalmente, en virtud de la cancelación de las reivindicaciones 22 a 27 el capItulo reivindicatorio modificado no se refiere a materia que se encuentre exenta de patentabilidad.

Por lo tanto, el capItulo reivindicatorio modificado se enmarca totalmente dentro de las exigencias establecidas por la Decision 486 de la Comunidad Andina.

8. Anexo me permito presentar copia de las pÆginas61 a 64, que corresponden al capItulo reivindicatorio, constituido por 33 reivindicaciones, en el cual se ha realizado la modificación expuesta anteriormente.

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REWINDICACIONES

1. Una proteina recombinante aislada caracterizada porque comprende SEQ ID NOS: 9, 13, 17, 21 6
5 25.

2. Una proteina recombinante aislada caracterizada porque comprende SEQ ID NO: 26 enlazada a N-2 repeticiones de SEQ ID NO: 27, en donde N es igual a un entero de 3 a 25.
10 3. La proteina de conformidad con la
reivindicación 2, caracterizada porque N es igual a un entero de 5 a 25.
4. La proteina de conformidad con la reivindicación 2, caracterizada porque N es igual a Un
15 entero de 10 a 25.
5. Una proteina recombinante aislada caracterizada porque comprende SEQ ID NO: 26 mÆs SEQ ID NO:
28 mÆs [N-2 repeticiones de SEQ ID NO: 271 mÆs SEQ ID NO:
29, en donde N es igual a un entero de 10 a 25.
20 6. Un poiinucleótido aislado caracterizado porque
comprende una secuencia de Æcido nucleico que codifica para
la proteina de conformidad con la reivindicaciÆn 1.
7. Un poiinucleótido aislado caracterizado porque
comprende una secuencia de Æcido nucleico que codifica para
25 la proteina de conformidad con la reivindicación 2.
8. Un polinucleótido aislado caracterizado porque comprende una secuencia de Æcido nucleico que codifica pare
la proteina de conformidad con la reivindicaciôn 3.

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9. Un polinucleótido aislado caracterizado porque
comprende una secuencia de Æcido nucleico que codifica para
la protelna de conformidad con la reivindicación 4.
10. Un poliriucleótido aislado caracterizado
5 porque comprende una secuencia de Æcido nucleico que codifica pera la protelna de conformidad con la
reivindicación 5.
11. Una proteina recombinante aislada
caracterizada porque comprende SEQ ID NOs: 7, 11, 15, 19
10 23.
12. Un polinucleótido aislado caracterizado
isporque comprende una secuencia de Æcido nucleico que codifica pare la protelna de conformidad con la
reivindicación 11.
15 13. El po.linucleôtido de conformidad con la
reiv–ndicación 6, caracterizado porque comprende SEQ ID
NOS: 8, 12, 16, 20 6 24.
14. El polinucleótido de conformidad con la reivindicacidn 12, caracterizado porque comprende SEQ ID
20 NOS: 6, 10, 14, 18 6 22.
15. Un polinucleótido aislado caracterizado
porque comprende un polinucleótido que tiene al menos 80%
de identidad con SEQ ID NOS: 6, 10, 14, 18 6 22 sobre la longitud complete de la secuencia.
25 16. El polinucleótido de conformidad con la
reivindicacióri 15, caracterizado porque comprende un polinucleótido que tiene al menos 90% de identidad.
17. El polinucleótido de conformidad con la reivindicacidn 15, caracterizado porque comprende un
30 polinucleótido que tiene al menos 95% de identidad.

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18. El polinucleótido do conformidad con la reivindicación 15, caracterizado porque comprende on polinucieótido que tiene al menos 99% de identidad.

5 19. La proteina de conformidad con las
reivindicaciones 1, 2 11, caracterizada porque la
proteina es 0-eniazada con -(1-3)--Gal-GalNac.
20. Una composicidn caracterizada porque
comprende una cantidad terapØuticamente efectiva de la
10 proteina de conformidad con la reivindicación 19 en un vehiculo farmacØuticamente aceptable.
21. La composición de conformidad con la reivindicación 20, caracterizada porque comprende ademÆs hialuronanno o hilano.
15 22. Un vector de expresión caracterizado porque
comprende ci poiinucieótido de conformidad con las reivindicaciones 6 6 7 enlazado operablemente a una secuencia do control de expresión.
23. Un mØtodo para producir una proteina
20 recombinante, caracterizado porque comprende:
cultivar cØlul&s transformadas con el vector do
expresidri do conformidad con la reivindicaciôn 22 en medic
de cultivo liquido y
recoger la proteina recombinante del medic.
25 24. El mØtodo do conformidad con la
reivindioación 23, caracterizado porque recoger la proteina comprende:
concentrar la proteina al filtrar el medio a
travØs do una membrana;
30 recoger la proteina retenida de la membrana y

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WO 00/64930 PCT/USOO/10953 TR1BQECT[NS
This application is a continuation-in-part of patent application U.S. Serial No.
09/298,970, filed on April 23, 1999, the entire contents ofwhich is hereby incorporated by

reference.
5 Background ofthe Invention
The invention relates to lubrication of mammalian joints.
Osteoarthritis (OA) is the one ofthe most common form ofjoint disease. Factors
which contribute to the development of OA include a family history of OA, previous damage
to the joint through injury or surgery, and age ofthe joint, i.e., "wear and tear" ofthe
10 articulating surfaces ofthe joint. OA is very common in older age groups, but can affect

children as well.

Current treatment is directed to relieving pain and other symptoms of OA, e.g., by
administering analgesics and anti-inflammatory drugs. Other therapeutic approaches include viscosupplementation by administering hyaluronic acid and derivatives thereof to joint tissue
15 to increase the viscosity ofsynovial fluid.
Summary ofthe Invention
The invention features a novel treatment for osteoarthritis and other degenerative joint
diseases by tribosupplementation. Tribosupplementation is carried out by administering
lubricating polypeptides directly to the injured or arthritic joint. Unlike viscosupplementation,
20 tribosupplementation does not substantially increase the viscosity ofthe solution, e.g.,
synovial fluid, to which it is added. The viscosity Of a solution to which a tribonectin is added increases no more than 10%, preferably no more than 5%, more preferably no more than 2%,
more preferably no more than 1%. Most preferably, the viscosity ofthe solution to which a tribonectin is added is unaltered or decreases.

25 Accordingly, the invention provides a tribonectin. A tribonectin is an artificial boundary lubricant which contains at least one repeat of an amino acid sequence which is at
least 50% identical to KEPAPTT (SEQ ID NO:3). A tribonØctin comprising at least one 0-
linked lubricating moiety. Preferably the lubricating moiety is a (l-3)Gal-GalNAc moiety,
The amino acid sequence ofthe protein backbone. of a naturally-occurrring tribonectin may
30 differ depending on alternative splicing of exons ofthe human megakaryocyte stimulating
factor (MSF) gene. For example, the tribonectin contains amino acids 1 to 24 and 200 to 1404
of SEQ ID NO:l but lacks amino acids 25-199 of SEQ ID NO: l. The tribonectin contains
amino acids 1 to 156 and 200 to 1404 of SEQ ID NO: 1 but lacks amino acids 157 tol 99 of
SEQ ID NO:!. The tribonectin contains amino acids I to 106 of SEQ ID NO: I and 200 to

WO 00/64930 PCT/USOO/10953 1404 of SEQ ID NO:! but lacks acids 107 to 199 of SEQ ID NO: 1. The tribonectin contains amino acids ito 25 of SEQ ID NO: 1, 67 to 106 of SEQ ID NO:1 and 200-to 1404 of SEQ ID NO: 1 but lacks amino acids 26 to 66 of SEQ ID NO:!. Tribonectins are purified naturally-occurring polypeplides or sythetically produced or recombinant polypeptides. The amino acid sequence ofthe backbone of synthetic or rcombinant tribonectins is at least 50% identical to
the amino acid sequence of a naturally-occurring tribonectin and possess at least 50% ofthe lubricating activity of a naturally-occurring tribonectin.;’
A tribonectin is formulated for admiriistratkm to a mammalian joint or other tissue,
eg., cardiac tissue, for which lubrication is desired. Preferably, the tribonectin is a
10 recombinant or chemically-synthesized lubricating polypeptide, For example, a tribonectin
includes a substantially pure polypeptide the amino acid sequence of which includes at least
one repeats but less than 76 repeats. Each subunit contains at least 7 amino acids (and

typically, 10 or fewer amino acids). The amino acid sequence of each subunit is at least 50% 40
identical to SEQ ID NO:3, and a non-identical amino acid in the reference sequence is a
15 conservative amino acid substitution. For example, one or both ofthe threonine residues are
substituted with a serine residue. Preferably, the amino acid sequence ofthe subunit is
identical to SEQ ID NO-3. The tribonectin may also contain one or more repeats ofthe amino
acid sequence XXTTTX (SEQ ID NO:4). Polypeptides or other compounds described herein
are said to be "substantially pure" when they are within preparations that are at least 60% by
20 weight (dry weight) the compound ofinterest. Preferably, the preparation is at least 75%,
more preferably at least 90%, and most preferably at least 99%, by weight the compound of
interest. Purity can be measured by any appropriatestandaid method, for example, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.
Where a particular polypeptide or nucleic acid molecule is said to have a specific
25 percent identity to a reference polypeptide or nucleic acid molecule of a defined length, the
percent identity is relative to the reference polypeptide or nucleic acid molecule. Thus, a
peptide that is 50% identical to a reference polypeptide that is 100 amino acids long can be a
50 amino acid polypeptide that is completely identical to a 50 amino acid long portion ofthe reference polypeptide. It can also be a 100 amino acid long-polypeptide which is 50%
30 identical to the reference polypeptide over its entire length.
A polypeptide or nucleic acid molecule which is "substantially identical" to a given reference polypeptide or nucleic acid molecule is a polypeptide or nucleic acid molecule
having a sequence that has at least 85%, preferably 90%, and more preferably 95%, 98%, 99%
or more identity to the sequence ofthe given reference polypeptide sequence or nucleic acid

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molecule. "Identity" has an art-recognized meaning and is calculated using well known
published techniques, e.g., Computational Molecular Biology, 1988, Lesk A.M., ed., Oxford University Press, New York; Biocomputing: Informatics and Genome Projects, 1993, Smith, D.W., ed., Academic Press, New York; Computer Analysis of Sequence Data, Part I, 1994,
5 Griffin, A.M. and Griffin, E.G., eds., Eumana Press, New Jersey; Sequence Analysis in
Molecular Biology, 1987, Heinje, G., Academic Press, New 20 York; and Sequence Analysis Primer, 1991, Gribskov, M and Devereux, J, eds, Stockton Press, New York) While there
exist a number of methods to measure identity between two polynucteotide or polypeptide sequences, the term "identity" is well known to skilled artisans and has a definite meaning
10 with respect to a given specified method. Sequence identity described herein is measured
using the Lasergene software package (DNASTAR, Inc. Madison, WI). The MegAlign
module used is the Clustal V method (Higgins et al. 1989, CABIOS 5 (2):151-3. The
parameters used are gap penalty 10, gap length penalty 10.
Alternatively, nucleic acids which differ from a given reference sequence hybridize at
15 high stringency to a stand of DNA having the reference sequence or the complement thereof.
High stringency conditions are hybridization at 42 degrees C in 50% formamide, a first wash
at 65 degrees C in 2 X SSC and a second wash at 65 degrees C in 0,2 X SSC.
A tribonectin is characterized as, reducing the coefficient offriction (.i) between
bearing surfaces. For example, reduction of frictioh ismeasured in vitro by detecting a
20 reduction in friction in a friction apparatus using latex glass bearings Reduction offriction is
also measured in vivo, e.g., by measuring reduction of patient pain. Tribonectins ofthe
invention are lubricating compositions. Polypeptides that have at least 50% (but less than
100%) amino acid sequence identity to

reference sequence are tested for lubricating function
by measuring a reduction in the t between bearing surfaces.
25 A tribonectin includes an 0-linked oligosaccharide, e.g., an N-acetylgalactosamine and galactose in the form beta (1-3)Gal-GaINAC. For example, KEPAPTT (SEQ ID NO:3) and XXTTTX (SEQ ID N0:4) repeat domains are glycosylated by beta (1-3)Gal-Ga1NAC (which
may at times be capped with NeuAc in the form of (1-3)Gal-GaLNAC-NeuAc. The term "glycosylated" with respect to a polypeptide means that a carbohydrate moiety is present at
30 one or more sites ofthe polypeptide molecule.’ For example, at least 10%, preferably at least
20%, more preferably at least 30%, and most preferably at least 40% ofthe tribonectin is glycosylated Up to 50% or more ofthe tribonectin can be glycosylated Per cent
glycosylation is determined by weight

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A tribonectin polypeptide contains a substantially pure fragment of(MSF. For
example, the molecular weight of a substantially pure tribonectin having an amino acid sequence of a naturally-occurring tribonectin is in the range of 220-280 kDa. Preferably, the apparent molecular weight of a tribonectin is less than 230 kDa, more preferably less than 250
kDa, and most preferably less than 280 kDa. A protein or polypeptide fragment is defined as a polypeptide which has an amino acid sequence that is identical to part, but not all, ofthe
amino acid sequence of a naturally-occurring protein or polypeptide from which it is derived,
e.g., MSF The tribonectin may contain a polypeptide, the amino acid sequence ofwhich is at
least 50% identical to the sequence of residues 200-1140, inclusive, of SEQ ID NO:l, e.g., it
10 contains the amino acid sequence of residues 200-1140, inclusive, of SEQ IDNO:1. In
another example, the polypeptide contains an amino acid sequence that is at least 50%
identical to the sequence ofresidues 200-1167, inclusive, of SEQ ID NO:l, e.g., one having the amino acid sequence identical to residues 200-1167, inclusive, of SEQ ID NO:!. The
polypeptide contains an amino acid sequence that is at least 50% identical to the sequence of
15 residues 200-1212, inclusive, of SEQ ID NO:!, e.g., the amino acid sequence ofresidues 200-
1212, inclusive, of SEQ ID NOel, or the polypeptide contains an amino acid sequence that is at
least 50% identical to the sequence ofresidues 200-1263, inclusive, of SEQ ID NO:!, e.g., an
amino acid sequence identical to residues 200-1263:, inclusive, of SEQ ID NO:!. Preferably,
the sequence ofthe polypeptide lacks the amino acid sequence ofresidues 1-24, inclusive, of
20 SEQ ID NO:l and/or the amino acid sequence ofresidues 67-104, inclusive of SEQ ID NO:l.
The invention also features an isolatedriucleic acid molecule encoding a tribonectin.
For example, the nucleic acid includes a sequendthat is at least 50% identical to nucleotides 631-3453, inclusive, of SEQ ID NO:2. Preferably, the nucleic acid encodes a polypeptide
with the amino acid sequence of residues 200-1140 of SEQ ID NO: 1. For example, the
25 nucleic acid has a nucleotide sequence identical to that of nucleotides 631-3453, inclusive, of
SEQ ID NO:2, or a degenerate variant thereof. An isolated nucleic acid molecule is a nucleic
acid molecule that is separated from the 5’ and 3’ coding sequences or non-coding sequences
with which it is immediately contiguous in the naturally occurring genome of an organism.
Isolated nucleic acid molecules include nucliic acid molecules which are not naturally
30 occurring, e.g., nucleic acid molecules created by recombinant DNA techniques. For example,
the nucleic acid ofthe invention includes nucleotides 631-3453, inclusive, of SEQ ID NO:2,
but not nucleotides which are immediately contiguous to those sequences in the naturally-
occurring genomic sequence or naturally-occurring eDNA

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Also within the invention is a method of lubricating a mammalian joint by contacting
the joint with purified MSF or a tribonectin. The mammal is preferably a human, horse, dog,
ox, donkey, mouse, rat, guinea pig, cow, sheep, pig, rabbit, monkey, or cat, and the joint is an articulating joint such as a knee, elbow, shoulder, hip, or any other weight-bearing joint.
5 Tribonectins are administered intra-articularly. Therapeutic joint lubrication is also carried out
by gene therapy, e.g., by contacting the joint or synovial fluid with a nucleic acid encoding a tribonectin. For example, nucleic acids are administered to a synovial cavity by intra-articular injection.
In addition to functioning as a boundary lubricant in a mammalian joint, a tribonectin
10 is used as a boundary lubricant between soft mammalian tissues such as skin or internal organs
or between a mammalian tissue and a medical device such as a prosthetic implant.
Accordingly, the invention encompasses a biocompatible composition containing a tribonectin
in a form suitable for the inhibition oftissue adhesion formation For example, the tribonectin
is in the form of a film, membrane, foam, gel, or fiber. The term "film," as used herein, means
15 a substance formed by compressing a foam or gel to a thin membrane, e.g., by casting into a
flat mold and air drying to a thin membrane, or by compressing a gel or fibers, or by allowing
or causing a gel or fibers to dehydrate. The term "foam," as used herein, means a substance
with a porous structure formed, e.g., by introduction of as air into a tribonectin solution,
suspension, gels, or fiber. The term "bioabsorbable," as used herein, refers to the ability of a
20 tissue-compatible material to degrade in the body after implantation, into nontoxic products
which are eliminated from the body or metabolized. A "biocompatible" substance, as the term
is used herein, is one that has no medically unacceptable toxic or injurious effects on
biological function. Tribonectin compositions for the prevention of adhesions are also
formulated as compositions suitable for extrusion, e.g., to form a mold upon which tissue can
25 grow without adhering.
A method inhibiting adhesion formation between a first surface and a second surface in
a mammal is carried out by placing a tribonectin btveen the first and second surfaces in an
amount sufficient to prevent adhesion ofthe surfaces in the mammal. For example, one or
both ofthe surfaces is a mammalian tissue, and a tribonectin placed between them prevents
30 formation of adhesions during the healing process. Alternatively the first or the second
surface (or both) is an artificial device such as an orthopedic implant. Tissues to be treated
include those injured by surgical incision or trauma.
Also within the invention is a method for diagnosing osteoarthritis or a predisposition
thereto by obtaining a biological sample from a mammal and measuring the amount of an

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MSF fragment in the biological sample. An increase in the amount compared to a control,
e.g., a predetermined value associated with a negative diagnosis or a biological sample from a mammal known to be free of osteoarthritis, indicates that the mammal suffers from
osteoarthritis or is predisposed to developing osteoarthritis. Any biological sample is suitable
5 for testing in the diagnostic method; typically, the biological sample is synovial fluid, blood,
serum, or urine. Preferably, the MSF fragment contains the amino acid sequence of SEQ ID
NO:3. Alternatively, the MSF fragment contains the amino acid sequence of EPAPTT (SEQ
IL) NO: 5,- a product oftrypsin cleavage of tribonectin) or the amino acid sequence of
PTTKEP (SEQ ID NO:6; a product of elastase cleavage of a tribonectin).
10 Other features and advantages ofthe invention will be apparent from the following description ofthe preferred embodiments thereof, and from the claims.
Brief Description ofthe Drawing

Fig. 1 is a diagram of a sequence alignment ofthe amino acid sequence ofMSF and a 40
human tribonectin. MSF exons 7, 8, and 9 are shown in their entirety; exon 6 was abbreviated
15 due to its length (940 amino acids). Exon 6 contains a total of 76 repeats ofthe degenerate
sequence KEPAPPT (SEQ ID NO: 3) which function as sites for 0-glycosylation. C-terminal
lysine/arginine residues ofsequenced tryptic fragments are shadowed. Amino acids 214-222
and 300-309 correspnding to exon 6-specific forward and reverse primers are shadowed and
underlined. The amino acid sequence coordinates refer to the amino acid sequence of SEQ ID
20 NO: 1.

Figs. 2A-B are diagrams dfphenotypic isoforms oftribonectin expression by human synovial fibroblasts and articular chondrocyts, respectively. Fig. 2A graphically shows MSF
exons expressed in tribonectin isoforms. Fig. 2B schematically shows exon expression in
articular chondrocytes compared to synovial fibf6lIsts Exons 2, 4, and 5 are alternatively
25 spliced resulting in isoform expression:
Fig. 3 is a diagram showing the alternative splicing boundaries of tribonectin isoforms
VI, V2, and V3.

Detailed Description
30 A number of naturally-occurring tribonectin isoforms wer identified and isolated. For example, a human lubricating polypeptide was purified from synovial fluid and found to
contain amino acids encoded by exons 2 and 4-12 ofthe MSF gene (but not exons I or 3).
The gene encoding naturally-occurring full length MSF contains 12 exons, and the naturally-occurring MSF gene product contains 1404 amino acids with multiple polypeptide sequence
6

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synovial fluid that confers lubricating capabilities to the articular joint. The boundary
lubricant isolated from human synovial fluid contains amino acids encoded by exons 2, and 4-
12 ofthe MSF gene, i.e., the alternative splice variant lacks amino acids encoded by exons I
and 3 ofthe MSF gene A recombinant or chemically-produced polypeptide containing at
5 least exon 6 (but not exons 1 or 3) ofMSF is usefMl to prevent and/or treat osteoarthritic disease. A recombinant or chemically-produced lubricating polypeptide containing at least
one repeat ofthe amino acid sequence KEPAPTT (SEQ ID NO: 3) either identically or with conservative substitution is also administered to lubricate mammalian joints.
Production and Purification ofRecombinant Lubricating Polyneptides
10 To produce recombinant polypeptides, DNA containing exon 6 of MSF (nucleotides 631-3453 of SEQ ID NO:2) in an appropriate expression vector is transfected into a cell.
The DNA can also contain some or all of exon 7 (nucleotides 354-3534 of SEQ ID NO:2),
exon 8 (nucleotides 3535-3670 of SEQ ID NO:2), or exon 9 (nucleotides 3671-3822 of SEQ
ID NO:2) ofthe MSF gene. Primers for polymerase chain reaction (PCR) methods to generate
15 DNA which spans various exons of MSF are shown below, Other isoforms are expressed by cloning nucleic acids corresponding to the exons of MSF encoding amino acid sequences
sought to be expressed

T&516 4 PCR.Prirners

MSF exons Forward Primer Reverse Primer

Exon 2 5’AGATTTATCAAGCTGTGCA 5’TTTACAGGAAAGC
GGGAG3’ TCCGCAGTG3’

(SEQ ID NO: 7) (SEQ ID NO: 8)
Exori 6 5’TCAAGGTCACAACTCCTGA 5’CTCTCGGTAAGTAATC
CACG3’ CATGTCGG3’

(SEQ ID NO:9) (SEQ ID NO: 10)
Exons 2-12 5’TTGTTGCTGCTGTCTGTTTT 5’TGGATAAGGTCTGCCC
CG3’ AGAACGAG3’

(SEQ ID NO: 11) (SEQ ID NO: 12)
Exons 6-12 5 ’TCAAGGTCACAACTCCTGA 5’ GATGGTGTGTGTTTGA
CACG3’ GAAGGTCC3’

(SEQ ID NO: 13) .(SEQID NO: l4)
20

Methods of designing forward and re primer used to make DNAs which encode
tribonectin polypeptides of varying lengths and which incorporate various exons ofthe MSF
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used therapeutically for treatment or prevention of arthritic diseases. The polypeptides are
also used to raise antibodies against a naturally-occurring or recombinantly produced
10 lubricating glycoproteins or glycopeptides.
For example, the recombinant gene product is expressed as a fusion protein and
purified using a commercially available expression and purification system, e.g., the pFLAG expression system (IBI). The expression systems that may be used for purposes of the
invention include, but are not limited to, microorganisms such as bacteria (e.g., E. coil and B.
15 subtilis) transformed with recombinant bacteriophage DNA, plasniid DNA, or cosmid DNA
expression vectors containing the nucleic acid molecules described herein. For production of glycosylated polypeptides, eukaryotic expression systems are used Yeast (for example, Saccharornyces and Pichia) transformed with recombinant yeast expression vectors containing
the recombinant nucleic acid encoding a triboncirpolypeptide are used. Insect cell systems
20 infected with recombinant virus expression vectors (for example, baculovirus) containing the
nucleic acid molecules encoding a tribonectin and mammalian cell systems (e.g., COS, CEO,
BEK, 293, VERO, HeLa, MDCK, W138, and NIH 3T3 cells) harboring recombinant
expression constructs containing promoters derived from the genome of mammalian cells S(e.g.,the metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late
25 promoter and the vaccinia virus 7.5K promoter) are also useful. In addition to clinical
applications, recombinant polypeptides are injected into a rabbit or rodent to produce
antibodies as described below.
The synovial fluid of an inflamed or injured joint contains proteolytic enzymes that
degrade lubricating proteins or polypeptides. For example, infiltrating immune cells such as
30 neutrophils secrete trypsrn and/or elastase Even a minor injury to an articulating Joint or an
inflammatory state can result in cellular infiltration and proteolytic enzyme secretion resulting
in traumatic synovitis Synovitis for a period of a few days or weeks can result in the loss of
the cytoprotective layer of ajoint, which in turn leads to the loss of cartilage. Non-lubricated cartilaginous bearings may experience premature wear which may initiate osteoarthritis.
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Individuals who clinically present with a traumatic effusion (e.g., "water on the knee") are predisposed to developing osteoarthritis; the elaboration of proteolytie enzymes degrades and depletes naturally-occurring lubricating compositions in the synovial fluid. Depletion of
natural lubricating compositions occurs in other inflammatory joint diseases such as
5 rheumatoid arthritis. Replacing or supplementing the synovial fluid ofsuch injured joints with
the lubricating compositions ofthe invention prevents the development of osteoarthritis in the
long term (e.g., years, even decades later) and immediately lubricates the joint to minimize
short term damage.
Analogs, homologs, or mimetics of lubricating peptides which are less susceptible to
10 degradation in vivo are used to lubricate mammalian joints. Analogs can differ from the
naturally-occurring peptides by amino acid sequence, or by modifications which do not affect
the sequence, or both. Modifications (which do not normally alter primary sequence) include
in vivo or in vitro chemical derivitization of polypeptides, e.g., acetylation or carboxylation.
Also included are modifications of glycosylation, e.g., those made by modifying the
15 glycosylation patterns of a polypeptide during its synthesis-and processing or in further
processing steps, e.g., by exposing the polypeptide to enzymes which affect glycosylation,
e.g., mammalian glycosylating or deglycosylating enzymes.
Where proteolytic degradation ofthe peptide’s following injection into the subject is a
problem, replacement of a particularly sensiti.Łtideband with a noncleavable peptide
20 mimetic bond renders the resulting peptide more stable, and thus more useful as a therapeutic.
To render the therapeutic peptides less susceptible to cleavage by peptidases such as trypsin or elastise, the peptide bonds of a peptide may be replaced with an alternative type of covalent
bond (a "peptide mimetic"), Trypsin, elastase, and other enzymes may be elaborated by
infiltrating immune cells during joint inflammation. Trypsin cleaves a polypeptide bond on
25 the carboxy-side of lysine and arginine; elastase cleaves on the carboxy-side of alanine,
glycine. Thrombin, a serine protease which is present in hemorrhagic joints, cleaves a peptide
bond on the carboxy-side of arginine. Collagenases are a family of enzymes produced by fibroblasts and chondrocytes when synovial metabolism is altered (e.g., during injury). These enzymes cut on the carboxy-side of glycine and proline. One or more peptidase-susceptible
30 peptide bonds, e.g., those which appear in’h KEPAPTT (SEQ ID NO:3) repeat sequence, are
altered (e.g., replaced with a non-peptide bond) to make the site less susceptible to cleavage,
thus increasing the clinical half-life of the therapeutic formulation
Such mimetics, and methods of incorporating them into polypeptides, are well known
in the art. Similarly, the replacement Of an L-amino acid residue with a D-amino acid is a
12

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standard way ofrendering the polypeptide less sensitive to proteolysis. Also useful are amino-terminal blocking groups such as t-butyloxycarbonyl, acetyl, theyl, succinyl, methoxysuccinyl, suberyl, adipyl, azelayl, dansyl, benzyloxycarbonyl, fluorenylmethoxycarbonyl,
methoxyazelayl, methoxyadipyl, methoxysuberyl, and 2,4, -dinitrophenyl.
5 Clinical formulations oftribonectins may also contain peptidase inhibitors such as N-methoxysuccinyl-AlaAla-Pro-Val chioromethylketone (an inhibitor of elastase).
Other clinically acceptable protease inhibitors (e.g.,. as described in Berling et al., 1998, mt. J. Pancreatology 24:9-17) such as leupeptin, aprotinin, al-antitiypsin, a2-macroglobulin, al-protease inhibitor, antichymotrypsin (ACHY), secretory leukocyte protease inhibitor (PSTI)
10 are also co-administered with a tribonectin to reduce proteolytic cleavage and increase clinical
haiflife A cocktail oftwo or more protease inhibitors can also be coadrninistered
Compositions oftribonectin polypeptides or nucleic acids encoding the polypeptides
are formulated in standard physiologically-compatible excipients known in the art., e.g., phosphate-buffered saline (PBS). Other formulations and methods for making such
15 formulations are well known and can be found in, e.g., "Remington’s Pharmaceutical
Sciences". Tribonectins are also formulated with nóncrosslinked hyaluronic acid preparations
or viscosupplementation compositions, such as cross-linked hyaluronic acid preparations.
When a tribonectin is added to a viscosupplement formulation, the- interaction of the
tribonectin with hyaluronic acid reduces the viscosity ofthe viscosupplement.
20 Methods of making a glycopeptide and determining % glycosylation are known in the art, e.g., as described in U.S. Pat. No. 5,767,254. The presence ofN-acetylgalactosamine is indicative ofthe presence of 0-linked oligosaccharides (or Ser/Thr-linked oligosaccharides) in which Ga1NAc is commonly found in O-glycàsidic alpha-linkage directly to amino acid. The presence of 0-linked oligosaceharide is also detected by binding to Jacalin-Sepharose, an
25 immobilized plant lectin that binds to the core disactharide sequence Gal beta (1-3) GaINAc
linked to Ser/Thr in glycoproteins, or peanut agglutinin, which binds to beta (1-3) Gal-
Ga1NAC. 0-linked oligosaccharides are distinguished from N-linked oligosacharides using
standard methods. For example, oligosaccharides in 0-glycosidic linkage, but not in N-
glycosidic linkage, are susceptible to release from peptide by treatment with mild base in the
30 presence ofsodium borohydride (50 mM NaOH, 1M NaBH4, 16 hr at 45CC.) to cause a beta-
elimination reaction.
Veterinary Applications
Canine osteoarthritis is a prevalent clinical disorder that is treated using the methods
described herein. Osteoarthritis afflicts an estimated one in five adult dogs; an estimated 8
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million dogs suffer from this degenerative, potentially debilitating disease. Yet, many owners
do not recognize the signs of chronic canine pain. While any dog can develop osteoarthritis,
those most at risk are large breeds, geriatric dogs, very active dogs (such as working or
sporting animals), and those with inherited joint abnormalities such as hip or elbow dysplasia
5 Equine degenerative joint disease such as teoarthntis is a cause of lameness and
impaired performance in horses. As with humans

other mammals, degenerative joint
diseases which affect horses are progressive disorders ofsynovial joints characterized by
articular cartilage degeneration and joint effusion. Acute or chronic trauma, overuse, developmental disease, joint instability and old age leads to synovitis, impaired chondrocyte
10 metabolism, and the formation of fissures in the joint cartilage. Destructive enzymes such as
trypsin, elastase, stromelysin and hyaluronidase are released into the joint where they degrade synovial fluid and cartilage components, resulting in decreased synovial fluid viscosity, poor lubrication, depressed cartilage metabolism and enhanced wear resulting in pain and cartilage erosion. Current therapeutic approaches include medications for pain relief and anti-
15 inflammatory drugs. The compositions and methods described herein are useful to replenish
the lubricating capabilities ofthe affected joint.
Administration ofTherapeutic Polyeptide
Standard methods for delivery of peptides are used Such methods are well known to
those of ordinary skill in the an. For intra-articular administration, tribonectin is delivered to
20 the synovial cavity at a concentration in the range of 20-500 j.tg/ml in a volume of
approximately 0.1-2 ml per injection. For example, I ml of a tribonectin at a concentration of
250 g/nil is injected into a knee joint using a fine (e.g., 14-22 gauge, preferably 18-22 gauge) needle. The compositions ofthe invention are also useful for parenteral administration, such
as intravenous, subcutaneous, intramuscular, and intraperitoneal.
25 For prevention of surgical adhesions, the tribonectins described herein are administered in the form of gel, foam, fiber or fabric. A tribonectin formulated in such a manner is placed
over and between damaged or exposed tissue interfaces in order to prevent adhesion formation between apposing surfaces. To be effective, the gel or film must remain in place and prevent
tissue contact for a long enough time so that when the gel finally disperses and the tissues do
30 come into contact, they will no longer have: a tendency to adhere. Tribonectins formulated for
inhibition or prevention of adhesion formation (e.g.,. in the form of a membrane, fabric, foam,
or gel) are evaluated for prevention of post-surgical adhesions in a rat cecal abrasion model (Goldberg et al., In Gynecologic Surgery and Adhesion Prevention, Willey-Liss, pp. 191-204, 1993). Compositions are placed around surgically abraded rat ceca, and compared to non-
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treated controls (animals whose ceca were abraded but did not receive any treatment). A
reduction in the amount of adhesion formation in the rat model in the presence ofthe
tribonectin formulation compared to the amount in the absence ofthe formulation indicates
that the formulation is clinically effective to reduce tissue adhesion formation.
5 Tribonectins are also used to coat artificial limbs and joints prior to implantation into a mammal. For example, such devices are dipped orbathed in a solution of a tribonectin, e.g.,
as described in U.S. Pat. Nos. 5,709,020 or 5,702,456.
Lubricating polypeptides are at least about 10 ammo acids (containing at least one
KEPAPTT (SEQ ID NO 3) or at least one XXTTTX (SEQ ID NO 4) repeat), usually about 20
10 contiguous amino acids, preferably at least 40 contiguous amino acids, more preferably at least
50 contiguous amino acids, and most preferably at least about 60 to 80 contiguous amino acids
in length. For example, the polypeptide is approximately 500 amino acids in length and
contains 76 repeats of KEPAPTT (SEQ ID NO:3). The polypeptide is less than 1404 residues
in length, e.g., it has the amino acid sequence of naturally-occurring MSF (SEQ ID NO:1) but
15 lacks at least 5, 10, 15, 20, or 24 amino acids of naturally-occurring MSF. Such peptides are
generated by methods known to those skilled in the art, including proteolytic cleavage of a recombinant MSF protein, de novo synthesis, or genetic engineering, e.g., cloning and
expression of at least exon 6, 7, 8, and/or 9 ofthe MSF gene.
Tribonectin polypeptides are also biochemically purified. The enzyme chymotrypsin
20 cleaves at sites which bracket amino acids encoded by exon 6 of the MSF gene. Thus, a
polypeptide containing amino acids encoded by exon ofthe MSF gene (but not any other MSF exons) is prepared from a naturally-occurring or recombinantly produced MSF gene product
by enzymatic digestion with chymotrypsin The polypeptide is then subjected to standard
biochemical purification methods to yield a substanialIy pure polypeptide suitable for

ek 25 therapeutic administration, evaluation oflubricating activity, or antibody production.
Therapeutic compositions are administered in a pharmaceutically acceptable carrier
(e.g., physiological saline). Carriers are selected on the basis ofmode and route of
administration and standard pharmaceutical practice. A therapeutically effective amount of a
therapeutic composition (e.g., lubricating polypeptide) is an amount which is capable of
30 producing a medically desirable result, e.g., boundary lubrication of a mammalian joint, in a
treated animal. A medically desirable result is a reduction in pain (measured, e.g., using a
visual analog pain scale described in Peyron et al., 1993, J. Rheumatol.(suppl.39):10-15) or increased ability to move the joint (measured, e.g., using peddmetry as described in Belcher et
30 al., 1997, J. Orthop. Trauma 11:106-109). Another method to measure lubricity of
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small volume of synovial fluid from the
affected joint and test the lubricating properties in,vitro using a friction apparatus as described herein.
As is well known in the medical arts, dosage for any, one animal depends on many
factors, including the animal’s size, body surface iea, age, the particular compound to be administered, sex, time and routeofadrninistrÆtion general health, and other drugs being administered concurrently. Administration is generally local to an injured or inflamed joint. Alternatively, the polypeptides are administered via a timed-release implant placed in close proximity to a joint for slow release at the site of an injured or inflamed joint.
10 Gene Therany
Gene therapy is carried out by administering to a mammal a nucleic acid encoding a
therapeutic lubricating polypeptide, e.g., DNA encoding one or more repeats or the amino acid sequence KEPAPTT (SEQ ID NO:3) or DNA encoding a lubricating fragment of MSF, by
standard vectors and/or gene delivery systems. Suitable gene delivery systems include
15 liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes
viruses, retroviruses, adenoviruses and adeno-associated viruses.
In addition to a gene delivery system as described above, the therapeutic composition

may include a pharmaceutically acceptable carrier, e.g,, a biologically compatible vehicle such
as physiological saline, suitable for administration b an animal A therapeutically effective
20 amount of a nucleic acid or polypeptidŁcomposition is an amount which is capable of
producing a medically desirable result in a treated animal, e.g., a reduction in pain associated
with joint movement, an increase in lubricating function of synovial fluid.
Parenteral administration, such as intravenous, subcutaneous, intramuscular, and
intraperitoneal delivery routes, may be used to deliver the compound. Preferably, therapeutic
25 compositions such as nucleic acids or polypeptides are delivered iritra-articularly. Dosage for
any one patient depends upon many factors, including the patient’s size, body surface area,
age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs (e.g., anti-inflammatory drugs, viscotherapeutic drugs) being
administered concurrently. A preferred dosage for administration of nucleic acids is from
30 approximately 106 to 1022 copies ofthe nucleic acid molecule.
DNA is he introduced into target cells ofthe patient by standard vectors, e.g., a vector which contains DNA encoding a trihonectin operably linked to a promoter sequence Suitable
gene delivery systems may include liposomes, receptor-mediated delivery systems, naked
DNA, and viral vectors such as herpes viruses, rtrovimses, and adenoviruses, among others.
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DNA may be administered locally using an adenovirus or adeno-associate virus
delivery system using standard methods. For example, methods of delivering DNA
intraarticularly to synovial fluid and methods of delivering DNA to cells from synovial fluid
(e.g., synovial fibroblasts or chondrocytes) are described in U.S. Pat. No. 5,858,355. The only
5 cis-acting sequences required for replication and packaging ofrecombinant adeno-associated
virus (AAV) vector are the AAV terminal repeats. Up to 4 kb of DNA is inserted between the terminal repeats without effecting viral replication or packaging. To package a recombinant
AAV vector, a plasmid containing the terminal repeats and DNA encoding a therapeutic polypeptide is co-transfected into cells with a plasmid that expresses AAV rep and capsid
10 proteins. The transfected cells are then infected with adeno-associated virus, and recombinant
AAV virus containing the desired sequences is isolated from cells approximately 48-72 hours
after transfection. Recombinant virus is then administered for gene therapy applications using known methods.

Electroporation is another method of introducing DNA into target cells, e.g., synovial
15 fibroblasts or ehondrocytes, ex vivo. Cells to be electroporated are placed into Hepes buffer
saline (EBS) at a concentration of about 107cells per ml. The DNA to be electroporated is
added at a concentration of approximately 5-20 micrograms/ml of HBS. The mixture is placed
into an electroporation device and an electric field is applied according to standard protocols,
e.g., in a range of between about 250 and 300 volts. Following introduction of DNA into
20 synovial cells ex vivo, the genetically modified autologous synovial cells are transplanted back

into the donor by intra-articular injection. Approximately 107 cells are injected intra-
articularly into joints in a volume of approximately I ml.
Synovial cells into which DNA is introduced are obtained using routine methods, e.g.,
through an arthroscope. The arthroscope is a small, hollow rod inserted into the knee via a
25 small puncture wound which allows access to a surgical instrument to recover synovial cells
arthroscopically. In some cases, the synbvial cells in arthroscopicafly excised tissue are
aseptically recovered by enzymatic digestion ofthe connective tissue matrix. For example, the synovium is cut into pieces of approximately 1 mm diameter and digested sequentially with
trypsin (0.2% w/v in Gey’s Balanced Salt Solution) for 30 minutes at 37°C, and collagenase
30 (0.2% w/v in Gey’s Balanced Salt Solution) for 2 hours at 37°C. A suspension of genetically-
modified cells is injected into a recipient.mammalian joint. Intra-articular injections ofthis
type are routine and carried out in the doctor’s office without additional surgical intervention.
Repeat injections are carried out as needed.

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pharmaceutical carrier and injected intra-articularly. Gene therapy is also administered as a prophylactic measure to prevent the development of osteoarthritis in those individuals
determined to be highly susceptible of developing this disease, e.g., those who have suffered
5 an acute joint injury. Direct intra-articular injection of a DNA encoding a therapeutic
polypeptide into a joint results in transfection ofthe recipient synovial cells to allow
expression of DNA.
Drugs which stimulate an endogenous tribonectin promoter, e.g, TGF-beta, may also
be administered as described above to increase the level ofsynovial expression.
10 Production of Antibodies Specific for Synvial Lubricating Polypei,tides
Antibodies specific for lubricating polypeptides are obtained by techniques well known
in the art Such antibodies can be polyclonal or monoclonal Polyclonal antibodies can be
obtained, for example, by the methods described in Ghose et al., Methods in Enzymology,
Vol. 93, 326-327, 1983. For example, a lubricating polypeptide encoded by nucleotides 632-
15 3453 of SEQ ID NO:2 is used as an immunogen to stimulate the production of polyclonal
antibodies in the antisera of a rabbit. Similar methods can be used to raise antisera in animals
such as goats, sheep, and rodents.
Monoclonal antibodies are obtained by the well known process described by Milstein
and Kohler in Nature, 256:495-497, 1975, or as modified by Gerhard, Monoclonal Antibodies,
20 Plenum Press, 1980, pages 370-371. Hybridomas are screened to identify those producing
antibodies that are highly specific for a synovial lubricating polypeptide. Preferably, the
antibody has an affinity of at least about 108 liters/mole

more preferably, an affinity of at
least about 109 liters/mole. Monoclonal or polyclonal antibodies provide a means ofrapidly purifying large quantities ofrecombinant lubricating pdlypeptides.
25 In addition to antibodies which are directed to the peptide core of a tribonectin, an antibody directed to a sugar portion otb a g1ydop ide cOmplex of a tribonectin is desirable.
To generate an antibody to the peptide core, a peptide spanning amino acids 200-350 of SEQ
ID NO:l is used. Shorter peptides, e.g., 8-15 amino acids in length, which are identical to an
8-15 amino acid portion of amino acids 200-350 of SEQ ID NO:l are also used to generate
30 such antibodies. Other peptides to be used as immunogens for antibodies specific for the
peptide core of a tribonectin include those which are in the region of amino acids 24-66 of
SEQ ID NO:!, amino acids 105-155 of SEQ ID NO:l, or amino acids 156-199 of SEQ ID
NO:l. To generate antibodies which bind to a glycosylated tribonectin polypeptide (but not a deglycosylated or nonglycosylated form), the immunogen is preferably a glycopeptide, the
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amino acid sequence of which spans

highly g1yc6ylated portion of a thbonectin, e.g., a
peptide with an amino acid sequence ofresidues 200-1140 of SEQ ID NO:l, Shorter glycopeptides, e.g., 8-15 amino acids in length, within the same highly glycosylated region are
also used as immunogens. Methods of generating antibodies to highly glycosylated
S biomolecules are known in the art, e.g., as described by Schneerson et al., 1980, J. Exp. Med.
152:361-376.

Methods of Diagnosis
Osteoarthritis is a disease that develops slowly and is difficult to diagnose until its late
stages when joint pain often compels an individual to seek medical treatment. Early diagnosis
10 of osteoarthritis or a predisposition to develop the disease allows early intervention to prevent
or reduce the development of advanced osteoarthritis. The invention provides methods of
early detection ofthis disease or a predisposition to develop it by tasting bodily fluids such as
serum or urine for the presence offragments of naturally-occurring trihonectins or the
presence offragments of MSF Detection and quantitatlon ofsuch peptides in biological
15 fluids is well known in the art, For example, a standard sandwich ELISA assay is carried out
using two different antibodies (e.g., a first antibody which binds to an oligosaccharide portion
ofthe glycopeptide and a second antibody which binds to the peptide core ofthe glycopeptide)
to a naturally-occurring tribonectin. Alternatively, standard protein sequencing by liquid chromatography and mass spectroscopy, as is described below, is used to detect MSF
20 fragments in biological samples. A control value is a predetermined value associated with a
negative diagnosis; alternatively, a control sample is a biological sample from a mammal
known to be free of osteoarthritis. An increase in the amount compared to a 30 control value
or sample indicates that the mammal suffers from osteoarthritis or is predisposed to
developing osteoarthritis.
25 Characterization of a Tribonectin from Human Synovial Fluid
Aliquots ofsynovial fluid from patients undergoing diagnostic arthroscopy and total
knee replacement were collected and assayed in the friction apparatus. In both cases, the
synovial fluid was aspirated prior to initiation o -surgical procedure and immediately centrifuged at 10,000 x g at 4°C for 2 hrs to remove cellular debris. Samples which were
30 contaminated with blood were discarded. Aliquots with normal lubricating ability were
pooled and stored at -20C.
Purification and Isolation of a Tribonectin
Human synovial fluid (200 ml) was filtered through 0.22 micro sterile filter units
(Nalgene) at 4°C over two days. Retentate was scraped offfilter membranes and resuspended
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with 50 mM NaAc buffer, p1-1 5.5, to the original synovial fluid volume containing proteolytic inhibitors: 1 mM phenyhnethyl sulfonyl fluoride (PMSF), 1 mM parachloromercuricbenzoic
acid (PCMB), and 10 mM ethylenediarnine tetraacetate (EDTA). Digestion of hyaluronic acid
was carried out at 37°C by streptomyces hyaluronidasô

1 U/mi of resuspended synovial
5 fluid The digest was loaded on a DEAE column (Whatman International, Maidstone, UK)
settled volume of 300 ml, equilibratedwith NaAcbüffer, 50 mM and washed with 1.5 L ofthe
same buffer. The material with lubricating activity was eluted off ofthe DEAE matrix with 1
M NaCl. A 1 L wash was collected and concentrated via a 500 ml Amicon flow cell with an XM-100 membrane (mw cutoff 100 kDa). The concentrated sample was dialyzed against 25
10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.5 mM CaCl2.
The DEAE-bound concentrate was loaded onto a peanut agglutinin (PNA)-agarose
affinity column with a settled bed volume of 25 ml, equilibrated at room temperature with 25
mM phosphate and 0.15 NaCl buffer, pH 7.4. Unbound protein was eluted with the same
buffer until absorbance at 230 and 280 nm decreased to background. Material with lubricating
15 activity was maximally eluted in the presence of a step-wise gradient of or-lactose at a
concentration of 0,07 M in 25 mM Tris and 0.15 M NaCl at pH 7.4. This material was loaded
onto an Actigel ALD agarose (Sterogerie Bioseparations, Arcadia, CA) coupled via amine
groups to a murine monoclonal antibody against human fibronectin (Zymed Laboratories Inc., San Francisco, CA) to remove fibronectrn as a contaminant Eluted material was assayed for
20 purity on SDS-PAGE (5-15% acrylamide) stained with Coomassie blue and by HPLC.
Protein electrophoresis standards were from GibcoBRL (Grand Island, NY), and DNA
ladder standard was from FMC Bioproducts (Rockland, ME).
High Pressure Liquid Chromatography
A Bondpak C18 3.9 x 150 mm column (Waters, Milford, MA) was eluted in reverse
25 phase with 45% (v/v) methanol (Sigma) and 5% (v/v) acetonitrile (Aldrich) HPLC grade at 1
ml/min at 35°C. The eluate was assayed by a photo diode array detector PDA 996 (Waters),
and material in peak fractions were analyzed by purity plots calculated using Milleniurn 32
software (Waters).
Friction Apparatus
30 A standard friction apparatus (e.g., an apparatus described by Jay et al., 1992, Conn. Tiss. Res. 28:71-88 or Jay et al., 1998, J Bibmed, Mater. Res. 40:414-418) was used to
measure lubricating activity. Natural latex was oscillated against a ring of polished glass with
a constant contact area of 1.59 cm2. The learingsistem was axially loaded within a gimbals
system free to rotate around two perpendicular horizontal axes. Latex and glass as bearing
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flat surface with small asperity heights on the order
of 0.05 mm Latex, like cartilage, is compliant Within the gimbals system, these surfaces possess near perfect co-planarity. Accordingly, fluid wedges were not generated and only a thin layer of boundary fluid was present. The entraining velocity (i.e., sliding speed) was 0.37 mm/sec with a constant contact pressure of 0.35 x 106 N/rn2.
The friction apparatus recorded displacements ofthe gimbals system around the
vertical loading axis through a linear displacement voltage transducer, the output voltage of
which was directly proportional to the magnitude ofthe frictional torque. The, peak to peak amplitude ofthis signal was related to x by a previous calibration with known frictional
10 torque.
Test surfaces were cleaned extensively before use. A 3.8 x 3.8 cm piece oflatex
strapped onto the stainless steel stud was washed under running distilled deionized water
(DDW) for 2 mm. It was then placed in a shallow bath of 0.9% NaC1 physiological saline
(PS). The glass slide was scrubbed with a 1% (v/v) 7x detergent (Flow Laboratories, McLean,
15 VA) solution in IJDW for 10 mm and then allowed to soak in the same solution at 100°C A 5
min. scrubbing was also performed with the hot 7X solution followed by rinsing for 2-4 mm
under running DDW
The i was measured at 35°C and was preceded by a baseline measurement ofthe i
with PS. Lubrication was manifested by a reduction of i relative to the t of PS. Negative
20 delta values indicate lubrication, whereas positive values indicate friction. Addition of 200
iii of PS and later 200.i1 oftest lubricant was followed by bringing the bearing surfaces close enough so that the solution wet both surfaces. After 5 mm for equilibration, the latex-coated bearing was brought to rest on the glass as it was oscillating. Peak to peak voltages were automatically recorded after 1, 3 and 5 mins. At this point, the surfaces were separated for 2

25

and then brought back together for another 5 mm session. The 3 and 5

t values of

the last two 5 mm. sessions typically stabilized and were recorded.
Human serum fibronectin was purchased from Sigma Chemical (F0895, St. Louis,
MO) and dialyzed against PS before use in: the friction apparatus.
Boundary lubricants exert their effect by changing the physmco-chemical characteristics
30 of a surface Bearing surfaces must generate a mutual repulsion in order to be lubricated in the
boundary mode. Typical room temperature examples of boundary lubricants are graphite,
teflon and molybdenum sulphide. Such compositions reduce friction between bearing
surfaces, and therefore, are used as positive controls in assay to measure the lubricating

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properties oftribonectins. Tribonectins are boundary lubricants that can have an amphipathic character by coating non-biologic hydrophobic surfaces such as latex. The oligosaceharide component of a tribonectin networks with the surrounding aqueous environment. When the ultimate and penultimate sugars are removed from a naturally-occurring tribonectin purified
5 from synovial fluid, the lubricating ability is eliminated.
The latex:glass arthrotripsometer offers an expedient way to test purified biological
lubricating factors repetitively with reproducibility. Natural latex and polished glass represent bearing surfaces with little if any variation in physico-chemical characteristic from test to test.
By contrast, resected cartilage apposed to either polished glass or cartilage itself will
10 experience deformation that cannot be accurately controlled. The.’ observed in a cartilage-
cartilage bearing lubricated by synovial fluid was between 0.005 and 0.024. The values of p.
in the latex:glass system were appreciably higher and typically 0.04 or less. Differences in p. between the hearing materials are attributed to the 80% (wlw) water content of cartilage.
Protein Sequencing by Liquid Chromatography and Mass Spectrometry (LCMS)
15 Standard LCMS was carried out on tryptic digests ofthe purified lubricating material described above. Excised bands from 2 mm thick 5-20% gradient SDS-PAGE gels (Bio-rad Laboratories, Hercules, CA) containing the lubricating material was analyzed. The material
was deglycosylated by NaNase III and 0-glycosidase DS (Glyko, Novato; CA).
Deglycosylation was carried out with the above enzymes at activities of 0.17 U/mI and 0.10
20 U/mi, respectively, for 18 hrs in the presence of 0.5 mg/ml of a tribonectin purified from
synovial fluid. In all cases, the gel slices were cut through the middle of the band and were 16
MM in size. All contact surfaces were carefully cleaned with 50% (v/v) acetonitrile.
Sequence data was entered into the BLAST GENBANKfi search algorithm and matches

identified

25 Isolation and Culture of Human Synovial Fibroblasts
Human synovium with a normal appearance was obtained from a 30 year old white
male undergoing arthroscopy. Within 1 hr after surgery, the synovial tissue explant was
washed three times with Dulbecco’s calcium- and magnesium-free phosphate-buffered saline (GIBCO). Pieces 2 mm3 in size were placed in Dulbecco’s modification of Eagle’s medium
30 (GIBCO), supplemented with 100 U of penicillin and 100 p.g of streptomycin per ml
(GIBCO), containing 4 mg/ml of Clostridiopeptidase A (Worthington Biochemical CLS, 125-
200 U/mg) sterilized through a 0.22 mm filter (Nalge). The tissue fragments were further

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divided with scissors in a 100 mm plastic petri dish (Falcon) and incubated for 4 hrs in 20 ml of medium at 37°C in a moist atmosphere of 5% carbon dioxide and 95% air.
The digest was well mixed many times by aspiration S into and expulsion from a
Pasteur pipette. An equal volume of 095% trypsin and 0.02% EDTA in modified Puck’s
Saline A (GIBCO) were added and incubation continued for a further hour under the same conditions. The suspension was centrifuged 10 min at 400 x g at 23°C and washed three times
each with 40 ml of calcium- and magnesium-free phosphate-buffered saline. The pellet was suspended in modified Eagle’s medium (20 ml) supplemented with 10% fetal bovine serum
(Flow Laboratories), 100 U of penicillin, and 100 mg ofstreptomycin per ml. Two milliliters
10 ofthis final mixture were plated per 60 mm plastic petri dish (Falcon). Synovial fibroblasts
were grown to confluence and cells harvested. Human skin fibroblasts (American Type
Culture Collection (ATCC) Designation CCD-10995K; ATCC, Mannassas, VA) which served
as a control were also grown and harvested using the procedure described above.
RNA Extraction and RT-PCR Analyses
15 RNA from synovial and skin fibroblasts was purified by RNeasy mini-columns and reagents (Qiagen, Crawley, Ltd., UK). Contaminating genomic DNA was removed by DNAshreddcr and DNase (RNase free) (Qiagen) First strand cDNA was synthesized by
reverse transcription and PCR amplification using the following oligonucleotide primers.
MSF-.exon 6 forward primer 5’ -CCAAACCACCAGTTGTAGATGAAGC-3’ (SEQ ID
20 NO:15) and MSF-exon 6 reverse primer-GCGGAAGTAGTCTTCTCTTTTCCATCAG3’
(SEQ ID NO:16). These primers correspond to nucleotide position numbers 674-698 and 953-
926, respectively, of the human MSF gene (SEQ ID NO:2; GENBANKt accession number
U70136). Thermal cycling conditions were 42°C for 12 mii’is., 95°C for 10 mins., followed by
43 cycles between 94°C x 20 sees and 55-65°C x 30-90 secs. A final extension for 7 mins was
25 at 72°C (Perkin Elmer Biosysteins).
Alternative splice variant of MSF is a Tribonectin
A lubricating polypeptide was purified from human synovial fluid using standard
biochemical methods followed by affinity chromatography with peanut agglutinin. The final fraction, which solely possessed lubricating ability, contained a product with an apparent
30 molecular weight of 280 kDa Components with a molecular weight in excess of 280 kDi
were not observed LCMS performed on trypticIfragments from the 280 kDa excised band indicated the presence oftwo different proteins that matched in the BLAST search algorithm
to fibronectin precursor and MSF (GENBANKTM Accession No. U70136). Sequences of
MSF were identified from both native and deglycosylated lubricating polypeptides.
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Accordingly, the purification scheme was terminated with an anti-fibronectin column resulting in the elimination offibronectin as an impurity (as assayed by Cl 8 analytical 1-IPLC and purity plot analysis). In addition, lower molecular weight bands at 70 and 160 kDa on SDS-PAGE were absent from the purified tribonectin preparation eluting -from the anti-fibronectin
column. The purified tribonectin assayed in the friction apparatus was found to display
boundary lubricating activity similar to that of whole synovial fluid (Table 5). By contrast,
purified serum fibronectiri raised friction indicating that synovial fluid lubricating ability was mediated by the purified tribonectin.
Table 5: Friction coefficients for a tribonectin purified from human synovial fluid and
10 fibronectin (Mean +SD; N=3)

LUBRICANT* (PS**)
Tribonectin 0,047

.006 0.131 –.007 -0.084 –.004
HSFt 0.040

. 005

0.135 –.009 -0.095 –.011
Fibronectin 0.181 0.136 +0.045

.005

* Tested at a concentration of 250.tg/mI in PS * * Physiological saline.
15 t Post-mortem human synovial fluid
Furthermore, LCMS oftryptic fragments identified portions of exons 6 through 9 of
MSF, inclusively. Purified tribonectin reacted to peanut agglutinin indicating the presence of
beta (1-3) Gal-Ga1NAC oligosaccharides by virtue ofits purification. An increase in
electrophoretic mobility was observed after digestion with NaNase III and 0-glycosidase DS,
20 indicating that the purified tribonectin is highly glycosylated via 0-linked oligosaccharides.
The apparent molecular weight of deglycosylated tribonectin purified from synovial fluid was
120 kDa.

RT-PCR analysis was completed using primers specific for nucleotide sequences
encoding the N-terminal end of exon 6 of MSF. RT-PCR’s using human synovial fibroblast
25 RNA generated a 280 bp product, the predicted distance between the designed primers.
Similar experiments without reverse transcriptase did not generate this product indicating that
the RNA was free of genomic DNA Purified RNA from skin fibroblasts did not produce any product using the same primers.
MSF was first isolated from human monocytes; a 25 kDa fragment of MSF was found
30 to stimulate the development of megakaryocytes. MSF precursor protein is 1404 residues in
size and constructed from 12 exons. Exon 6 appears to encodes a centrally located mucin that
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is 940 residues in length. Exon 6 has homology to vitronectin, exons 2 and 3 appear
homologous to somatomedin }3 -like regions, and exons 8, 9 are similar to hemopexin-like
regions in vitronectin. Hemopexin is a serum heme scavenging protein that interacts with
hyaluronate.
5 A tribonectin purified from synovial fluid and an articular cartilage superficial zone
protein (SZP) purified from articular cartilage share sequence identity with MSF but differ in
their apparent molecular weights and amino aci sequences
Example 1: MSF Gene Expression by Human Synovial Fibroblasts
A naturally-occurring tribonectin was found to be expressed by human synovial
10 fibroblasts from a gene encoding MSF. Some, but not all, ofthe exons ofthe MSF gene are
represented in the tribonectin gene product. For example, the tribonectin contains sequences encoded by exons 6-9 ofthe MSF gene, but lacks sequences encoded by at least one exon of
the naturally-occurring MSF gene.
MSF gene expression was evaluated as follows. Gel slices from the 240 kDa apparent
15 molecular weight band of purified tribonectin from pooled human synovial fluids with normal
lubricating ability were digested with trypsin N-terminal sequencing of tryptic fragments was performed by liquid chromatography mass spectrometry and results compared to known
sequences in GenBank’rm. Digestion of purified tribonectin with 0-glycosidase DS and
NaNaselil was used to indicate the molecular weight ofthe core protein Lubricating ability
20 was assayed using a standard friction apparatus that oscillates natural latex against a ring of
polished glass. :
Two previously identified protein species were present in the final lubricating fraction
of human synovial fluid. Tryptic fragments from fibronectin precursor (FN) and
megakaryocyte stimulating factor (MSF) were both identified. A 100% match (E values range
25 0.31 0.047) was observed with MSF sequences specific for exons 6 though 9. MSF is 1104
amino acids in size with multiple functional domains- similar to vitronectin. This tribonectin reduced the coefficient offriction (.t) from 0.142 to 0.036 whereas purified serum fibronectin raised t from 0.136 to 0.181. Forward and reverse RT-PCR primers corresponding to amino
acid positions 214-222 and 300 309 of SEQ ID NO:1 were used to probe purified mRNA
30 from human synovial fibroblasts grown in vitro. A280 bp gene product was observed
consistent with the predicted amino acid sequence. The data described herein indicate that a naturally-occurring tribonectin is secreted by synovial fibroblasts via expression ofthe MSF

gene.

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The methods described below were used to characterized a lubricating component in
synovial fluid.
Human Synovial Fluid Collection.
Aliquots of synovial fluid from patients undergoing diagnostic arthroscopy and total
5 knee replacement were collected and assayed in the friction apparatus. In both cases, the
synovial fluid was aspirated prior to initiation of any surgical procedure and immediately centrifuged at 10,000 x g at 4°C for 2 hrs to remove cellular debris. Samples that were grossly contaminated with blood were discarded. Aliquots with normal lubricating ability were
pooled and stored at -20°C.
10 Purification and Isolation of Human Tribonectins

Human synovial fluid 200 ml was filtered through 0.22 gm sterile filter units (Nalgene)
at 4°C over two days. Retentate was scraped offfilter membranes and resuspended with 50
mM NaAc buffer, pH 5.5, to the original synovial fluid volume. The resuspension buffer
contained proteolytic inhibitors:, 1mM PMSF, 1 mM PCMB and 10 mM EDTA. Digestion of
15 hyaluronie acid was carried out at 37°C by streptomyces hyaluronidase at 1 U/ml of
resuspended synovial fluid The digest was loaded on a DEAE column (Whatmnan
International, Maidstone, UK) settled volume of 300 ml, equilibrated with NaAc buffer, 50
mM and washed with 1.5 L ofthe same buffer. The desired material was eluted from the
DEAE matrix with 1 M NaCl. A I L wash was collected and concentrated via a 500 ml
20 Amicon flow cell with an XM-1 00 membrane (mw cutof = 100 kDa), The concentrated
sample was dialyzed against 25 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and
0,5 mM CaCI7.
The DEAE-bound concentrate was loaded Onto a peanut agglutinin (PNA)-agarose
affinity column with a settled bed volume of 25 ml, equilibrated at room temperature with
25 25 mM phosphate and 0.15 NaCl buffer, pH 7.4. Unbound protein was eluted with the same
buffer until absorbance at 230 and 280 nm decreased to background. Desired material was maximally eluted in the presence of a step-wise gradient of ct-lactose at a concentration of
0.07 M in 25 mM Tris and 0.15 M NaCl at p1-I 7.4. Pre-purified tribonectin was loaded onto
an Actigel ALD agarose (Sterogene Bioseparations, Arcadia, CA) coupled via amine groups to
30 a murine monoclonal antibody against human fibronectin (Zymed Laboratories Inc., San
Francisco, CA). Eluted material was assayed on SDS-PAGE 5-15% stained with Coomassie
blue and by HPLC.

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A p.Bondapak C18 3.9 x 150 mm column (Waters, Milford, MA) was eluted in reverse phase with 45% (v/v) methanol (Sigma) and 5% (v/v) acetonitrile (Aldrich) HPLC grade at
1 mLlmin at 35°C. Eluate was assayed by a photo diode array detector PDA 996 (Waters) and
5 peaks analyzed by purity plots calculated using Millenium 32 software (Waters).
Friction Apparatus
A standard friction apparatus was used to measure lubricating activity as described

above
The

was measured at 35°C and was preceded by a baseline measurement ofthe.t
10 with PS. Lubrication was manifested by a reduction of.i. relative to the

of PS. Negative Ajt
values indicate lubrication whereas positive values indicate friction. Addition of200.i1 of PS
and later 200 j.d oftest lubricant was followed by bringing the bearing surfaces close enough
so that the solution wet both surfaces After 5 mm for equilibration, the latex-coated bearing
was brought to rest on the glass as it was oscillating. Peak to peak voltages were
15 automatically recorded after 1, 3 and 5 mins. At this point the surfaces were separated for 2
min and then brought back together for another 5 min session. The 3 and 5

values of
the last two 5 min sessions typically stabilized and were recorded.
LCMS

LCMS oftryptic digests was carried out as described above. Bands were excised from
20 2 mm thick 5-20% gradient SDS-PAGE gels (Bio-rad Laboratories, Hercules, CA) for further
analysis. Deglycosylation was carried out using NaNase HI and 0-glycosidase DS (Glyko,
Novato, CA) (at concentrations of 0.17 U/mI and 0.10 U/mi, respectively, for 6 hi-s in the
presence of 0.5 mg/ml of protein. In all cases, the gel slices were cut through the heart ofthe
band and were 16 mm3 in size. All contact surfaces were carefully cleaned with 50% (v/v)
25 acetonitrile. Sequence data was entered into the BLAST GenBankTM search algorithm and

matches identified.
RNA Extraction and RT PCR Analyses
RNA from synovial and skin fibroblasts wa purified by RNeasy mini-columns and
reagents (Qiagen, Crawley, Ltd., UK). Contaminating genomic DNA was removed by
30 DNAshredder and DNase (RNase free) (Qiagen). First strand cDNA was synthesized by
reverse transcription and PCR amplification using the following oligonucleotide primers (the relative positions ofthe MSF-specific primers are shown in Fig. 1): MSF-exon 6 forward
primer 5’-CCAAACCACCAGTTGTAGATGAAGC-3’ (SEQ ID NO: 15) and MSF-exon 6

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reverse primer 5’ -GCGGAAGTAGTCTTCTCTTTTCCATCAG-3’ (SEQ ID NO: 16). These primers correspond to base pair position numbers 674-698 and 953-926 respectively in GENBANKTM Acession number U70136 (human MSF). Thermal cycling conditions were
42°C for 12 mins, 95°C for 10 mins followed by 43 cycles between 94°C x 20 sees and 55 -
5 65°C x 30 - 90 sees. A final extension for 7 mins was at 72°C (Perkin Elmer Biosystems),
Miscellaneous Reagents
Human serum fibronectin was purchased from Sigma Chemical (F0895, St Louis,
MO) and dialyzed against PS before use in the friction apparatus. Protein electrophoresis
standards were from GibcoBRL (Grand Island, NY) and DNA ladder standard was from FMC
10 Bioproducts (Rockland, ME).
A tribonectin from from human synovial fluid was purified. The final fraction which
solely possessed lubricating ability primarily contained a product with an apparent molecular weight of 280 kDa, The fraction also contained minor low molecular weight components,
which were removed with the final antibody-based purification step. Components with a
15 molecular weight in excess of 280 kDa were not observed. LCMS performed on tryptic
fragments from the 280 kDa excised band indicated the presence oftwo different proteins that matched in the BLAST search algorithm to fibronectin precursor and MSF (GENBANKTM Accession No. U70136). Sequences of MSF were identified from both native and
deglycosylated tribonectin. Accordingly, the purification scheme was terminated with an anti-
20 fibronectin column resulting in the near elimination offibronectin as an impurity on the basis
of C18 analytical HPLC and purity plot analysis In. addition, the minor low molecular weight
bands at 70 and 160 kDa on SDS-PAGE were absºflt from the purified preparation eluting

25

30

from the anti-fibronectin column. Purified tribonectin assayed in the friction apparatus was
found to display boundary lubricating activity similar to that ofwhole synovial fluid (Table 6).
Table 6: Friction coefficient for a human tribonectin and fibronectin
LUBRICANTI- (PS*)
Tribonectin 0.035 0.142 -0.106
Fibronectin 0.181 0.136 +0.045

* Physiological saline.
Tested at a concentration of 250 gIml in PS.
In contrast, purified serum fibronectin raised friction indicating that synovial fluid lubricating ability was mediated by tribonectin.

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WO 00/64930 PCTIUSOO/10953 LCMS oftryptic fragments correctly identified portions of exons 6 through 9 of MSF inclusively. Fragments encoded by nucleic acid sequences at the beginning and end of exon 6 were found. The tribonectin reacted to PNA indicating the presence of (1 -3)Gal-Ga1NAC oligosaccharides by virtue ofits purification. In addition there was a very significant increase in electrophoretic mobility after digestion with NaNase III and 0-glycosidase DS indicating
that the tribonectin is highly glycosylated

O-linked oligosaccharides. The apparent
molecular weight of deglycosylated tribonectin was 120 kDa.
RT-PCR analysis was completed using primers specific for nucleotide sequences
encoding the N-terminal end of exon 6 of MSF. RT-PCR’s using human synovial fibroblast
10 RNA generated a 280 bp product, the predicted distance between the designed primers.
Similar experiments without reverse transcriptase did not generate this product indicating that
the RNA was free of genomic DNA. Purified RNA from skin fibroblasts did not produce any product using the same primers.
The data described herein indicate that the exons ofthe MSF gene are alternatively
15 spliced to express lubricating polypeptides in synovial joints. Synovial fibroblasts are one of
the the sources ofsuch tribonectins in joints. An isolated naturally-occurring tribonectin
described herein (Mr-280 kDa) and SZP (Mr-345 kDa) are different molecules as they differ
in apparent molecular weight by 65 kDa. It is possible that MSF is a precursor for both
molecules by virtue of differing expression of MSF exons by two independent cell lines that
20 populate the synovial cavity. Repetitive LCMS sequencing’ oftryptic digests did not identify
any ofthe exons which bracket exons 6 through 9 in MSF. A naturally-occurring tribonectin purified from bovine calf was found to have the same molecular weight as a tribonectin
purified from the human sources.
Boundary lubricants exert their effect by changing the physico-chemical characteristics
25 of a surface. Bearing surfaces must generate a mutual repulsion in order to be lubricated in the
boundary mode. Commonplace room temperature examples are graphite, teflon and
molybdenum sulphide. Tribonectins appear to have an amphipathic character by coating non-biologic hydrophobic surfaces such as latex. The extensive glycosylations are important to
network with the surrounding aqueous environment. When the ultimate and penultimate
30 sugars are removed the lubricating ability is eliminated. The possibility of hyaluronate
binding to SZP or a tribonectin via protein expressed by exon 9 supports a mechanism by
which hyaluronate plays a role in synovial fluid A hyaluronate polymer may serve to bind
many SZP or tribonectin molecules in tandem side-by-side, thus distributing shear Stress and stabilizing lubricant molecules.
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The primary structure of MSF precursor (alternatively spliced gene products such as
tribonectins) is different from that of MG2, statherine, and proline rich glycoprotein which are found in saliva and lubricate dental bearings. Only M02 has been tested in the present
latex:glass bearing and found to lubricate as effectively as a tribonectin. Both glycoproteins
5 share the same 0-linked (1-3)GaI-Ga1NAe moiety. These data indicate that the 0-linked
3(l-3)Gal-GalNAc moiety is important for boundary lubrication activity.
Enzymatic digestion experiments indicate that boundary lubrication provided by
synovial fluid is highly sensitive to trypsin. These experiments demonstrated that the removal
of lubricating ability by phospholipases (contaminated with very small amounts oftrypsin-like
10 proteases) could be eliminated by co-incubation with protease inhibitors, leupeptin and
aprotinin. Lysine and arginine comprises 13,5% and 1.3% respectively ofthe exon 6 product
which supports the observation oftrypsin sensitivity, even though this is the same region of tribonectin that is extensively glycosylated and could therefore prevent proteolysis. It is likely
that even minor inflammatory states that elaborate trypsin or elastase have deleterious effects
15 upon the lubricating ability of naturally-occurring tribonectins. Non-lubricated cartilaginous
bearings may experience premature wear which can initiate osteoarthritis. Such conditions are treated using the lubricating polypeptides, i.e., tribonectins, described herein.
Example 2: Homology of a Tribonectin and SZP: Products of MSF Gene Expression by
Human Synovial Fibroblasts and Articular Chondrocytes Localized to Chromosome 1q25
20 Expression oftribonectins was evaluated in primary human synovial fibroblasts and primary articular cartilage. RNA was purified from human synovial fibroblasts and articular chondrocytes grown in vitro from tissue explants obtained from subjects without degenerative
joint disease. RT-PCR was used with multiple complimentary primer pairs spanning the
central mucin expressing exon 6 ofthe MSF gene and individual exons on both the N- and C-
25 terminal sides of exon 6. Exons 2, 4 and 5 appear to be variably expressed by synovial
fibroblasts and articular chondrocytes. A lubricating polypeptide, a tribonectin, expressed
from various exons ofthe MSF gene is expressed b’ both chondrocytes and synovial
fibroblasts in vitro. Both a trobonectin and superficial zone protein (SZP), a related
proteoglycan, share a similar (but not identical) primary structure. Tribonectins and SZP also
30 differ in post-translational modifications with 0-linked oligosaccharidcs; tribonectins contain
0-linked oligosaccharides, whereas such post-translational modifications have not been
detected in SZP. The oligosaceharides are predominant in tribonectins; limited amounts of chondroitin and keratan sulfate are found in SZP. Since most ofthe MSF exons are involved
in the expression of a tribonectin, a strong homology to vitronectin persists. Screening of a
30

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human genome BAC library with a eDNA primer pair complimentary for exon 6 identified
two clones. Both clones were complimentary for chromosome 1q25 by in situ hybridization.
This locus was implicated in camptodactyl-arthropathy-pericarditis syndrome (CAP) by
genetic mapping. CAP, a large joint arthropathy, is associated with ineffective boundary
5 lubrication provided by synovial fluid. Accordingly, the tribonectins described herein are
useful to prevent and/or treat CAP.
The data described below addresses the following questions: 1) Which ofthe 12 MSF
exons are expressed by synovial fibroblasts and presumably result in tribonectin secretion into
the synovial cavity, 2) how does this differ from the expression ofMSF by articular
10 chondrocytes which results in SZP secretion onto the surface of cartilage, 3) do tribonectins
and SZP share the same primary structure, 4) are tribonectins post-translationally modified
with chondroitin sulfate- a feature of SZP, and 5) does an isolated exon 6 product possess
boundary lubricating ability.
Human synovial fluid was collected and processed as described above, and tribonectins
15 were purified and isolated from human tissue using methods described above, Lubricating
activity was measured using a standard friction apparatus.
Immunodetection ofChondroitin-6-Sulfate

Monoclonal antibody M621 (ICN, Aurora, OH) was use to detect the presence of chondroitin-6-sulfatc in purified tribonectin. Standard dot and Western blotting techniques
20 were used.

Digestion of a tribonectin with Chymotryin
TLCK-treated chymotrypsin 0.1BTEE U/mlof human synovial fluid was incubated for
1 hr at 37°C. Immediately following digestion lubricating activity was assayed in the friction apparatus. Additional digestions were carried out in the same way but also in the presence of
25 10 jig/mi leupeptin and 5 jtg/ml aprotinin in the event that commercial TLCK treatment
incompletely inactivated contaminating trypsin.
Isolation and Culture ofHuman ndrocytes
In addition to isolating human synovial fibroblasts as described above, human
chondrocytes were isolated. Cartilage from the femoral condyles and tibial plateaus of human
30 knee joints was obtained at autopsy from donors without known history ofjoint disease or
from healthy organ donors from tissue banks. Cartilage slices were Cut into 2-3mm3 pieces,
washed with DMEM (Whittaker MAB Bioproducts, Walkerville, MD) and treated for 15
with trypsin 10% (v/v) in a 37°C waterbath.. The tissues were transferred to DMEM, 5%

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Culture ofHuman Monocytes
Human monocytes CRL-9855 in suspenson were purchased from American Type
Culture Collection (Mannassas, VA). Cells were concentrated by centriæ.igation and then resuspended in a T75 flask to concentration between 0.2 and 1.0 x 106/ml with Iscove’s
modified Dulbecco’s medium supplemented with 10% fetal bovine serum that was not heat inactivated (Flow Laboratories), 0.03 mM thymidine, 0.1 mM hypoxanthine and 0.05 mM 2-
10 mercaptoethanol. As the monocytes multiplied, the culture was expanded to maintain the
above concentration range.
RNA Extraction and RT-PCR Analyses
RNA from human synovial and articular chondrocytes and monocytes were purified by
RNeasy mini-columns and reagents (Qiagen, Valencia,CA). Contaminating genomic DNA
15 was removed by DNAshredder and DNase (RNase free) (Qiagen). Complimentry DNA
synthesis was done by incubating at 25°C for 10 min for extension of hexameric primers by
reverse transcriptase, and synthesis of cDNA at 42°C for 12

and activation of Ampli
Taq/denaturation ofRNA-cDNA complex at 95C for 10

Thermal cycling condition was
43 cycles of 94°C, 20 secs for denaturation and 62°C, 60 sees, and a final extension at 72°C
20 for 7 mins (Perkin Elmer l3iosystems, Foster City, CA) RT-PCR products were analyzed by
3% NuSieve GTG agarose (FMC, Rockland, ME) gel e1etrophoresis stained with ethidium bromide. Bands not corresponding to expected RT-PCR product sizes were excised and
extracted from agarose (Qiagen).
BAC clone library screening
25 An E. coil BAC library containing the human genome was screened (Research Genetics Inc., Huntsville, AL) with a cDNA primer pair complimentary to MSF exon 6 by
PCR. Forward primer 5’-CCAAACCACCAGTTGTAGATGAAGC-3’ (SEQ ID NO; 15) and reverse primer 5’ GCGGAAGTAGTCTTCTCTTTTCCATCAG-3’ (SEQ ID NO: 16) complimentary to base pair position numbers 674-698 and 953-926 of SEQ ID NO:2,
30 Candidate E. coil clones were grown in LB agar containing 12.5 mg/ml chioramphenicol at
37°C for 16 his. Large construct DNA free of genomic DNA was purified by anion exchange
and exonuclease digestion (Qiagen Large-construct kit, Valencia, CA).
In situ hybridization ofBAC clone DNA and chomosomes
4. .

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Metaphase chromosomes were prepared from short term lymphocyte cultures from a normal individual according to established protocols. Two micrograms of BAC DNA from positive
clones were labeled using BioNick labeling kit (Life Technologies, MD) using biotin 14-dATP
in a nick translation reaction according to a protocol supplied by the manufacturer. After nick
5 translation, the reaction products were purified on.a 0-50 column lOOng oflabeled DNA and
20.tg of coti DNA were co-precipitated with ethanol. The DNA was recovered by
centrifugation and dissolved in 20xl of hybridization solution (Hybrisol vii, Oncor). Probe hybridization, washing, microscopy and mapping ofthe probes were performed according to standard procedures.
10 Dijaestion of Trihonectins with Chymotrypin and Assay for Lubricating Ability
A tribonectin was purified from pooled human synovial fluid with normal lubricating
ability defined as Ap <-0.06 and possessed an apparent molecular weight of 280 kDa. Terminating the purification with an anti-fibronectin step was carried out to remove
contaminating bands. This step also reduced the purity angle to < 6°. A tribonectin at a
15 concentration of 250 j.ig/ml lubricated the latex:glass bearing as effectively as post-mortem
synovial fluid from a subject without degenerative joint disease (Table 7).

Table 7. Friction coefficients for human a human triboneetiri and fibronectin

20 Lubricant j.t (PS) N
Tribonectint 0.047

0.131 –.007 0.084 –.004 3
.006
HSF* 0.040

0.135

.009 -0.095 –.011 3
.005

Chmyotrypsin 0.130

0.107 –.024 +0.02

.035 3
.041

Mean– SD

tTested at a concentration of 250 jig/ml in PS.
Physiological saline
25 *Post-mortem human synovial fluid
§HSF digested with TLCK-treated chymotrypsin in the presence ofleupeptin and aprotinin.

Enzymatic digestions with TLCK-treated chymotrypsin were performed to determine
ifthe isolated exon 6 protein product possesses lubricating ability as this domain does not
30 contain aromatic amino acids The resulting digestate raised p. to 130 from the saline control
value p. = 107 Similar digestions in the preence of leupeptmlaprotinin continued to
demonstrate no lubricating ability (p.= +.02).
Immunodetection of Chondrotin-6-Sulfate
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Monoclonal antibody M621 C was used to probe tribonectins transferred to

nitrocellulose. No reaction was observed corresponding to the 280 kDa band on the original SDS-PAGE. This indicates that tribonectins do not contain chondroitin-6-sulfate.

RT-PCR Analysis of Synovial Fibroblast and Articular Chondrocyte RNA

5 RT-PCR of chondrocyte and synovial fibroblast mRNA was carried out using the

primers illustrated in Table 8. RT-PCR products ofthe predicted size in addition to others that

were smaller than predicted were observed for both cell lines. The primer pair spanning the C-

terminal end of exon 6 to the N-terminal end of exon 12 was observed to be 769bp, its

predicted length, for both cell lines. Similarly, the primer pair spanning the same position in

10 exon 6 to exon 11 produced an expected RT-PCR product of 654bp. The primer pair of exon 5

and 6 produced the expected RT-PCR product of 278bp

both cell lines.

S

Table 8: Primer pairs used in RT-PCR’s with RNA from human synovial fibroblasts and
15 articular chondrocytes.

Inter- Base Pairs (5 3’) Forward Base Pairs (5’

3’) Backward Primer
xon Primer
Primers
1-6 64-85 TTGTTGCTGCTGTCTGTT 819

796 GGGTCTGGGATTTATTG

TTCG (SEQ ID NO: 17) GTTTTGC (SEQ H) NO:23)
2-6 17-8-149 GGAGATGTGGGGAAGG 819 796 GGGTCTGGGATTTATTG
GTATTC (SEQ ID NO: 18) GTTTTGC (SEQ ID NO:24)
 6 371-394 CACCATCTTCAAAGAAA 819 796 GGGTCTGGGATTTATTG
GCACCTC (SEQ ID NO: 19) GTTTTGC (SEQ ID NO:25)
5-6 542-566 CCTCCTCTTCCTCTTCTT 819 796 GGGTCTGGGATTTATTG
CTTCAAC (SEQ ID NO:20): GTTTTGC (SEQ ID NO:26)
6-11 3427- GGCATTATCATCAATCC 4080- CACATTTGGAAGTCCTC
448 CATGC (SEQ INO21) 4057 TCCACAG (SEQ IDNO:27)
6 12 3427- GGCATTATCATCAATCC 4195- TTGCTCTTGCTGTTCTAC
448 CATGC (SEQ ID NO:22) 4171 TAGUCAC (SEQ ID NO:28)

Base pair position numbers correspond to accession numberU 70136 in GENBANKTM

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Table 9:. Primer pairs used in RT-PCR’s with RNA from human monoçytes.

Inter- Base (5’

3’) Forward Base (5’

3’) Backward Primer
exon Pairs Primer Pairs
Primers
 3 64-85 TTGTTGCTGCTGTCTGT 313

CATACTTCTTACATTGG

TTTCG (SEQ ID NO:29) 291 GCGTCG (SEQ ID NO:32)
1-4 64-85 TTGTTGCTGCTGTCTGT. 375- TGGTGGTGATGTGGGAT
TTTCG (SEQ ID NO:30) 354 TATGC (SEQ ID NO:33)
2-5 128- GGAGATGTCGGGAAGG 540- OGAGGAGGAGGACTCT
149 GTATTC (SEQ ID NO:31) 517 TG’ATTTTC (SEQ ID
N0L34)

Base pair position numbers correspond lo accession number U 70136 in

5 GENBANKTM.

The primer pair spanning from exon I to exon 6 failed to singularly generate the
predicted product size of 756 bp. Instead three smaller sized (350, 450 and 490bp) bands were observed for both cell lines. The primer pair spanning from exon 2 to the exon 6 N-terminus
10 produced a product roughly 420bp in size for both cell lines and was 272bp (692bp - 420bp)
shorter than its predicted size The predicted RT-PCR 692 bp product was faintly observed for chondrocytes and less so for synovial fibroblasts. The exon 2 to 6 primer pair 420bp product
was excised and sequenced which revealed the absence of exon 5 and most of exon 4 except
for the first two N-terminal amino acids Ala’° and Leu106 of SEQ ID NO 1) Sequencing ofthe
15 490 and 450bp RT-PCR products from.the exon 1 to 6 primer pair identified the same
deletions. Sequencing ofthe smaller 350bpproduct from the exon I to 6 primer pair revealed
these same deletions in addition to exon 2 except for the -terminal Q5. The primer pair of
exon 4 and 6 produced the expected RT-PCR product of 449bp for both cell lines and a
smaller 340bp product. Sequencing ofthis 340bp product revealed deletion of exon 5 except
20 for the N-terminal E’56

Alternative splicing was found to be responsible for generating 4 different
phenotypical isoforms of MSF from both synovial fibroblasts and chondrocytes (Figs. 2A and
28).
Phenotype VU consists of all 12 exons, V missing exon 5, V2 missing exon 5 and
25 most of 4, and V3 missing exon 5 and most of 4 and 2. Yet another isoform is a polypeptide
which contisn amino acids encoded by MSF exons 1 and 6-12 (missing exon 5); this is
likely the smallest naturally-occurring tribonectin. Each isoform contains the lubricating

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domain exon 6. The domain encoded by exon I plays a role in mediating binding ofthe tnbonectin to hydrophobic surfaces like cartilage
RT-PCR Analysis of Monocyte RNA
RT-PCRs with purified monocyte RNA using primer pairs (Table 9) complimentary to
5 MSF exon 6 did not produce any products. A primer pair spanning exons I to 3, produced a
PCR product of 250bp, its predicted size. Another primer pair spanning exons 1 to 4 produced
a PCR product that was approximately 1 3Obp smaller than the 31 2bp predicted size. Yet
another primer pair spanning exons 2 to 5 did not generate any PCR product, indicating that
exon 5 is not expressed. These data would appear to demonstrate that monocytes express
10 exons I through 3, 1 through 4 variably and do not express exon 6. The forward primer
sequence in exon 1 was common to all ofthe above RT-PCR work involving monocytes,
synovial fibroblasts and chondrocytes.
In situ hybridization ofBAC clone DNA and chromosomes
Screening of a human genome BAC clone library with the above MSF exon 6 eDNA
15 primer pair resulted in 2 candidate clones. BAC clones 330 and 285 hybridized to
chromosome 1 at q25 region (DAPI banding). To confirm the localization to chromosome 1, metaphase chromosomes were hybridized to BAC probes along with chromosome I specific centromere probe (VYSIS). Both ceætrorneric as’ell as the BAC probes hybridized to
chromosome 1. Subsequent G-banding ofthe same slides confirmed the q25 localization.
20 Synovial fibroblasts grown in Vitro alternatively spliced out either MSF exons 2,4 or 5, or a combination thereof. In the case of exon 4, expression stopped after the first two codons
ofthe exon (Ala 105 and Leu’°6). Isoforrns without exon 5 except for residue E’56 and one
without exon 2 except for Q25 also exist. Three RT-PCR products occurred with the exon 1 to
6 primer pair which appear to correspond to phenotypes Vi 3. However, the expected RT-
25 PCR 692bp product for the exon 26 primer pair was observed, indicating that an isoform VO,
which contains each ofthese exons also exists. It would appear that 4 or 5 different
phenotypical isoforms oftribonectins are expressed by synovial fibroblasts. Chondrocytes
share this phenotypic expression. A summary ofthe alternative splice sites for both cell lines
is illustrated in Fig. 3. The relatively greater intensity ofthe 692bp exon 2-6 and 449bp exon
30 4-6 primer products for chondrocytes suggests that these cells could potentially select for a
higher molecular weight isotype.
Exon 6 in MSF is positioned between anaiialogous heparin sulfate binding region
(MSF exon 4) and the hemopexin-like repeats (MSF exons 8,9). Separation ofthese domains encourages greater interaction with glycosaminoglycan which would differ from vitronectin in
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WO 00/64930 PCT/USOO/10953 which these domains are thought to compete for glycosanhinoglycan binding due to their proximity. Perhaps the ability to bind to glycosaminoglycan on either or both ends ofthe lubicating domain serves to stabilize lubricating activity on cartilage covered with the lamina splendins Alternatively, an interaction exists between a tribonectin and collagen type II,
which interaction serves to bind the tribonectin directly to cartilage.
Boundary lubricants such as tribonectins bind to bearing surfaces and generate a
mutual repulsion in order to lubricate in the boundary mode. This bifunctional attribute
appears as amphipathic behavior by coating non-biologic hydrophobic surfaces such as latex. Extensive glycosylations network with the surrounding aqueous environment. When ultimate
10 and penultimate sugars are removed the lubricating ability is eliminated. Expression of MSF
exon 6 is required for lubricting activity; optimal lubricting activity likely requires the
expression of a domain expressed by at least one additional MSF exon, e.g., exon 1. The chymotrypsin digestion experiment carried out was intended to excise the exon 6 product and
test this digestate for lubrication assuming that other released exon products would not
15 interfere with lubricating ability. The digestate did not lubricate we;;, despite replication in the
presence of leupeptin and aprotinin, suggesting that other exon products may be needed. For example, exon 3, which has a number of cysteine residues which are important in the
aggregation of mucin polymers, may be required. The ability of a tribonectin to coat a surface
is likely dependent upon hydrophobicity and the ability ofindividual tribonectin polymers to
20 aggregate.
Immunohistochemical studies failed to detect chondroitin-6-sulfate indicating that it is
not present or it is sterically hindered by 0-linked glycôsylations adjacent to D220EASG224
where chondroitin sulfate would be attached. The presence of chondroitin and keratan sulfate
in SZP was confirmed by 35S-labeling and release after digestion with keratanase and
25 chondroitin lyase ABC. However, no significant change in SZP molecular weight was
observed after this digestion, indicating that only a small amount ofsubstitution was likely. It
is possible that either glycosaminoglycan is conjugated to a tribonectin
Tribonectins and SZP are similar molecules sharing MSF exons 1,3 and 6 through 12,
and the alternatively spliced exons 2 through 5 Despite similar primary structure, differences
30 in post-translational modifications between the two proteins have been detected The presence
of the 0-linked lubricating moiety (l-3)Gal-GalNAc has not been detected for SZP as it has
for the tribonectins described herein. A difference in molecular weight between tribonectins
and SZP is supported by Western blotting with antibodies developed against SZP, which was purified from superficial zone chondrocytes.
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WO 00/64930 PCTIIJSOO/10953 Camptodactyl-arthropathy pericarditis syndrome (CAP)
In situ hydridization experiments indicated that DNA sequences encoding tribonectins
reside on chomosome 1q25, as both BAC clone probes independently localized to the same location. A boolean search of Online Mendelian Inheritance in Man (OMIM) revealed one
S arthritic disease which has been genetically mapped to the same locus. Camptodactyl-
arthropathy-pericarditis syndrome (CAP) is characterized by juvenile onset large joint
inflammatory arthropathy and congenital non-traumatic flexion contractures of one or more inter-phalangeal joints (camptodactyl).. The histopathologic appearance ofsynovial and teno-synovial tissues from patients with this autosomally.inherited disease is that of 13-type
10 synoviocyte hyperplasia and end-stage fibrosis CAP syndrome is a direct result ofineffective
lubrication resulting in premature joint wear. Some patients with CAP also develop
pericarditis. Isolated naturally-occurring or synthetic triboneetins are useful to treat CAP and
for lubricating pericardial tissues. CAP is diagnosed by detecting a mutation in the MSF gene.

15 Other embodiments are within the following claims.

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What is claimed is:

1. A tribonectin comprising at least one 0-linked lubricating moiety.
2. The tribonectin of claim 1, wherein said moiety is a 3(l-3)Gal-GalNAc moiety.
3. The tribonectin of claim 1, wherein said tribonectin comprises amino acids 1 to
5 24 and 200 to 1404 of SEQ ID NO:1, wherein said tribonectin lacks amino acids 25-199 of
SEQ ID NO: l.
4. The tribonectin of claim 1, wherein said tribonectin comprises amino acids Ito
156 and 200 to 1404 of SEQ ID NO 1, wherein said tribonectin lacks amino acids 157-199 of
SEQ ID NO: 1.
10 5 The tribonectin of claim 1, wherein said tribonectin comprises amino acids 1 to
106 of SEQ ID NO:1 and 200-1404 of SEQ ID NO: 1, wherein said tribonectin lacks amino
acids 107 to 199 of SEQ ID N0:1.
6. The tribonectin of claim 1, wherein said tribonectin comprises amino acids 1 to
25 of SEQ ID NO: 1, 67 to 106 of SEQ ID NO: 1 and 200-to 1404 of SEQ ID NO:1 wherein
15 said tribonectin lacks amino acids 26 to 66 of SEQ ID NO: 1.
7. A tribonectin comprising a polypeptide the amino acid sequence of which
comprises at least one but less than 76 subunits, wherein
(a) each subunit comprises at least 7 amino acids; and
(b) the amino acid sequence ofsaid subunit is at least 50% identical to SEQ
20 ID NO:3, wherein a non-identical amino acid is a conservative amino acid substitution.
8. The tribonectin of claim 7, wherein the amino acid sequence of said subunit is
SEQ ID NO:3.
9. The tribonectin of claim 7, wherein said tribonectin further comprises one or
more repeats ofthe amino acid seune of SEQ ID NO:4.
25 10. The tribonectin of claim 1, wherein said tribonectin is characterized as reducing
the coefficient of friction between bearing surfaces.
11. The tribonectin of claim 1’, wherein said tribonectin is characterized as
reducing the coefficient offriction between hearing surfaces in vitro.
12, The tribonectin of claim 1, wherein said tribonectin is characterized as reducing
30 the coefficient of friction between bearing surfaces in vivo.
13 The tribonectin of claim 1, wherein said tribonectin does not substantially
increase the viscosity of a solution to which it is added.
14. The tribonectin of claim 7, wherein said tribonectin comprises an 0-linked
oligosnecharide.
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14. The tribonectin of claim 7, wherein said tribonectin comprises an 0-linked
oligosaccharide.
15. The tribonectin of claim 14, wherein said oligosaccharide is an N-
acetylgalactosamine-galactose.
5 16. The tribonectin of claim 1, wherein at least 10% ofsaid tribonectin is
glycosylated.
17. The tribonectin of claim 1, wherein at least 40% ofsaid tribonectin is
glycosylated.
18. The tribonectin of claim 1, wherein the molecular weight ofsaid tribonectin is
10 in the range of 200-280 kDa.
19. The tribonectin of claim 1, wherein said polypeptide comprises a fragment of
megakai-yoeyte stimulating factor.
20. The tribonectin of claim 1, wherein said polypeptide comprises an amino acid
sequence that is at least 50% identical to the sequence ofresidues 200-1140, inclusive, of SEQ

15 IDNO:l.
21. The tribonectin of claim 1, wherein said polypeptide comprises the amino acid
sequence ofresidues 200-1140, inclusive, ofSEQflD N0:l.
22. The tribonectin of claim 1, wherein said polypeptide comprises an amino acid
sequence that is at least 50% identical to the sequence ofresidues 200-1167, inclusive, of SEQ

20 ID NO: 1.

23. The tribonectin of claim 1, wherein said polypeptide comprises the amino acid
sequence of residues 200-1167, inclusive, of SEQ ID NOel.
24. The tribonectin of claim 1, wherein said polypeptide comprises an amino acid
sequence that is at least 50% identical to the sequence ofresidues 200-1212, inclusive, of SEQ
25 IDNO:l.

25. ’I’he tribonectin of claim 1, wherein said polypeptide comprises the amino acid
sequence ofresidues 200-1212, inclusive, of SEQ ID NOel.
26. The tribonectin of claim 1, wherein said polypeptide comprises an amino acid
sequence that is at least 50% identical to the sequence ofresidues 200-1263, inclusive, of SEQ
30 ID NOel,
27. The tribonectin of claim 1, wheinaid polypeptide comprises the amino acid
sequence of residues 200-1263, inclusive, of SEQ ID NOel.
28. The tribonectin of claim 1, wherein said polypeptide lacks the amino acid
sequence ofresidues 1-24, inclusive, of SEQ ID NOel.
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29. The tribonectin of claim 1, wherein said polypeptide lacks the amino acid
sequence ofresidues 67-104, inclusive of SEQ ID NO I
30. An isolated nucleic acidinoleculeŁæcodinga tribonectin.
31. The nucleic acid of claim 30, wherein said nucleic acid comprises the sequence
5 of nucleotides 631-3453, inclusive, of SEQ IDNO:2.
32. A method of lubricting a mammalian joint, comprising contacting said joint
with an isolated MSF gene product.
33. A method oflubricating a mammalian joint, comprising contacting said joint
with the tribonectin of claim 1.
10 34. The method of claim 33, an articulating joint of a human.
35. The method of claim 33, an articulating joint of a dog.
36. The method of claim 33, an articulating joint of a horse.
37. The method of claim 33, wherein said joint is wherein said joint is wherein said
joint is wherein said tribonectin is administered intra-articularly.
15 38 A method oflubricating a mammalian joint, comprising contacting said joint
with the nucleic acid of claim 30

39. A method of preventing or treating camptodactyl-arthropathy-pericarditis
syndrome in a mammal, comprising administering to said mammal a tribonectin.
40. A biocompatible composition comprising a tribonectin, wherein said
20 composition is in a form suitable for the inhibition oftissue adhesion formation.
41. The composition of claim 40, wherein said tribonectin is in the form of a
membrane, foam, gel, or fiber.
42. A method inhibiting adhesion formation between a first surface and a second
surface in a mammal, said method comprising placing a tribonectin between said first and
25 second surfaces in an amount sufficient to prevent adhesion ofsaid surfaces in said mammal.
43. The method of claim 42, wherein said first surface and said second surface are
both injured tissues ofsaid mammal.
44. The method of claim 42, wherein said first or said second surface is an artificial
device
30 45. The method of claim 44, wherein

artificial device is an orthopedic implant.
46. The method of claim 42, whercinsaid tribonectin is in the form of a membrane,
foam, gel, or fiber.
47. The method of claim 42, wherein said injury is due to a surgical incision.
48. The method of claim 42, wherein said injury is due to trauma.
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49. The method of claim 42, wherein said first surface or said second surface is
pericardial tissue.
50. A method for diagnosing an osteoarthritis or a predisposition thereto in a
mammal, comprising measuring the amount of amgakaryocyte stimulating factor (MSF) or a
5 fragment thereof in a biological sample derived from said mammal, wherein an increase in said amount compared to a control indicates that said mammal suffers from osteoarthritis or is predisposed to developing osteoarthritis.
51. The method of claim 50: wherein said biological sample is synovial fluid,
blood, serum, or unite.
10 52. The method of claim 50, wherein said MSF fragment comprises the amino acid
sequence of SEQ ID NO:3.
53. The method of claim 50, wherein said MSF fragment comprises the amino acid
sequence of SEQ ID NO:5.
54. The method of claim 50, wherein said MSF fragment comprises the amino acid
15 sequence of SEQ IDNO:6.

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO DIRECCION DE NUEVAS CREACIONES

2020
Bogota, D.C.,

Abogado
ALICIA LLOREDA RICAURTE
(Apoderado)

ASUNTO Radicación 06-013.004
TrÆmite 002
Evento 001

S ActuaciOn 519
Oficio I
Folios 04

Patente de Invención Patente de Modelo de UtlIldad

Via Nacional

Via PCT (fase nacional) fl Cap.l JW Cap. II

No. Sol. Internacional PCT/US2004/026508
Fecha de Presentación Internal. 13 de agosto de 2004

Coma resultado del examen de fondo, realizado con fundamento en el artIculo 45 de la Decision 486 de la Comision de Ia Comunidad Andina, se le comunica

1. Documentos estudiados

DescripciOn: Folios: 32 a 92 como se radicó inicialmente

Reivindicaciones: Folios: 93 a 97 coma se radicó inicialmente
Folios: 196 a 199 como se radicO el 01/06/10.

Secuencias: Folios: 98 a 137 coma se radicó inicialmente

Prioridad: Folio: 1
Fecha: 14/08/2003
Pals: US
Nümero. 60/495741

Rta. Del solicitante: Folios: 190 a 195 coma se radicó el 01/06/10.

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2. Objeto de la invención

Titulo de Ia soticitud (folio 1): "MolØculas de lubricina recombinantes y usos de
las mismas".

La presente solicitud se refiere a nuevas molØculas de lubricina
recombinantes y a su uso como lubricantes, agentes antiadhesivos yb
suplementos intra-articulares.

El problema planteado en esta solicitud es la necesidad de proporcionar
composiciones terapØuticas para incrementar la lubricaciôn en articulaciones
y contrarrestar la indeseable adherencia entre tejidos.

S.. Para solucionar este problema tØcnico la solicitud en estudio proporciona una

serie de protelnas aisladas, el potinucleótido y las composiciones de las
molØculas de lubricina.

La Clasificación Internacional de Patentes (IPC) es: A61K38/17, 31/728,
39/395, C07K 14/435, A61 19/02,

3. De la solicitud de Patente

Reivindicaciones (artIculo 30 D 486):

El capitulo reivindicatorio de la solicitud en estudio contiru:ia presentando los siguientes defectos:

De la lectura de las reivindicactones no son claras las caracteristicas tecntcas
que comparten las protemnas reivindicadas entre si, asi como los polinucleOtidos y por ende las composiciones.

AdemÆsde lo anterior, se han introducido nuevas reivindicaciones que no cumplen con los requisitos de claridad dado que definen la materia sobre la base de caracterIsticas funcionales y resultados a alcanzar. (p.e. Reivindicaciones 26, 28, 29, 30)

Por lo anterior, el capItulo reivindicatorio NO cumple con el requisito de definición, claridad, concision o sustento por la descripciOn de acuerdo al art. 30 de la DecisiÆn486.y/o art.22 Tratado PCT.

4. Determinación del Estado de, IaTØcnica

La fecha que se ha tenido en cuenta para determinar el Estado de la TØcnica es:

Fecha de presentaciOn de la solicitud prioritaria: 14 de Aciosto de 2003.

27?-

Ten iendo en cuenta la fecha indicada se realizó la büsqueda en el estado de la tØcnica de documentos relacionados con la solicitud encontrÆndose los siguientes documentos mÆs relevantes:

NO Documento Titulo Fechade..
* publicacion
Dl W000/64930 TRIBONECTINS 02/11/2000

5. AnÆlisis depatentabilidad (artIcu!o 14 D486)

5.1.Novedad (articulo 16D486)

La reivindicación I y sus dependientes divulgan una protelna recombinante aislada caracterizada porque comprende SEQ ID NOS: 9, 13, 17, 21.

Para el anÆlisisde novedad, es necesario tener en cuenta que las caracterIsticas tØcnicas esenciales que comprenden las protelnas recombinantes definidas en las reivindicaciones, se remiten a la repeticiôn de la secuencia KEPAPTT y presentan actividad lubricante, tal como ha sido, ademÆs, argumentado por el solicitante en su respuesta del 01/06/2010.

Dl enseæa(ver folios: 200 a 222) un mØtodo para suplementar tribonectinas a articulaciones con deficiencias de Østas, en las cuales, se involucran molØculas caracterizadas porque comprenden secuencias KEPAPTT y presentan actividad lubricante de la misma forma que las reivindicadas en la solicitud, Tales molØculas se basan en la repetición de 1 a 76 veces dicha secuencia (pÆgina2 lInea 10 - 12), con fragmentos alternativos degenerados de la misma provenientes de la estructura natural de la lubricina dependiendo del corte alternativo de exones en el gen del factor estimulador de megacariocitos (Tablas l,3y4).

Los elementos caracteristicos de los pØptidos reivindicados ya eran conocidos, como se evidencia en Dl, asI como la forma de realizar dichas molØculas de lubricina, las posibles sustituciones y los resultados esperados. Portal razón, los compuestos reivindicados no pueden ser considerados novedosos.

De esta forma se establece que las reivindicaciones I a 33 no cumplen con el requisito de Novedad.

6. Conclusion:

Los requisitos de patentabilidad de la presente solicitud se encuentran afectados asI:

PN, Documento N ReivindicÆciofles Requisito que afecta afectadas
Dl W000/64930 1 -33 Novedad

t:2:

En cumplimiento del artIculo 45 de la Decision 486 de la ComisiOri de la Comunidad Andina, se le indica que debe dar respuesta dentro del tØrmino de 60 dIas contados
a partir de la fecha de notificaciOn de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de Ia respuesta subsistieran los impedimentos para la concesiôn, se denegarÆ la patente.

Notifiquese el presente oficio conforme a Ia dispuesto en el numeral 5.2, literal e), del capItulo quinto del tItulo primero de la Circular LJnica No 10 de 2001.

If

ALIX CARMENZA CESPEDES DE VERGEL
Directora de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha

CONCEPTO TECNICO EXAMINADOR TECNICO
No. 2764 COdigo No. 202089