Eficàcia dels tocotrienols com a estratègia de tractament de la fibrosi intestinal

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EFICÀCIA DELS TOCOTRIENOLS COM A ESTRATÈGIA DE TRACTAMENT DE LA FIBROSI INTESTINAL

Tesi doctoral presentada per

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LLISTAT D'ABREVIATURES

α-SMA, smooth muscle actin alpha

Apaf-1, Apoptotic peptidase activating factor 1

ATG16L, Autophagy related 16 like 1

bFGF, basic Fibroblasts Growth Factor

CARD15, Caspase Recruitment Domain family, member 15

CED-4, Cell death protein 4

CsA, ciclosporina A

CU, Colitis Ulcerosa

CXC3R1, Chemokine (C-X-C motif) 3 Receptor 1

FIH, Fibroblasts Intestinals Humans

FN, Fibronectina

FRT, Fracció Rica en Tocotrienols

HGF, Hepatocyte Growth Factor

HSC, Hepatic Stellate Cells

IGF, Insulin like Growth Factor

IL, Interleucina

IRGM, Immunity Related GTPase family, M

LAP, Latency Associated Peptide

LTBP, Latent TGF-β-binding Protein

MC, Malaltia de Crohn

MEC, Matriu extracel·lular

MII, Malaltia Inflamatòria Intestinal

MMP, Matrix Metalloproteinase

NOD2, Nucleotide binding Oligomerization Domain containing 2

PDGF, Plateled Derived Growth Factor

PSC, Pancreatic Stellate Cells

SC, Stellate Cell

SNP, Single Nucleotide Polymorphism

TEM, Transició Epiteli-Mesènquima

TGF-β, Transforming Growth Factor β

TIMP, Tissue Inhibitor of Metalloproteinases

TNBS, Trinitrobenzene sulfonic acid

TNF-α, Tumor Necrosis Factor alpha

Tsp-1, Trombospondina 1

TTP, Tocopherol Transport Protein

TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling

ÍNDEX

INTRODUCCIÓ

1) MALALTIA INFLAMATÒRIA INTESTINAL

La Malaltia Inflamatòria Intestinal (MII) és un terme que s'utilitza de manera genèrica per a referir-se a malalties de presentació crònica que tenen un curs recurrent i són d'etiologia desconeguda. Inclou una àmplia gamma de manifestacions clíniques, la característica principal de les quals és una inflamació crònica en algun punt del tub digestiu. El terme MII engloba bàsicament la colitis ulcerosa (CU) i la malaltia de Crohn (MC), encara que hi ha altres malalties que cursen amb inflamació intestinal i poden tenir manifestacions clíniques similars (Figura 1).

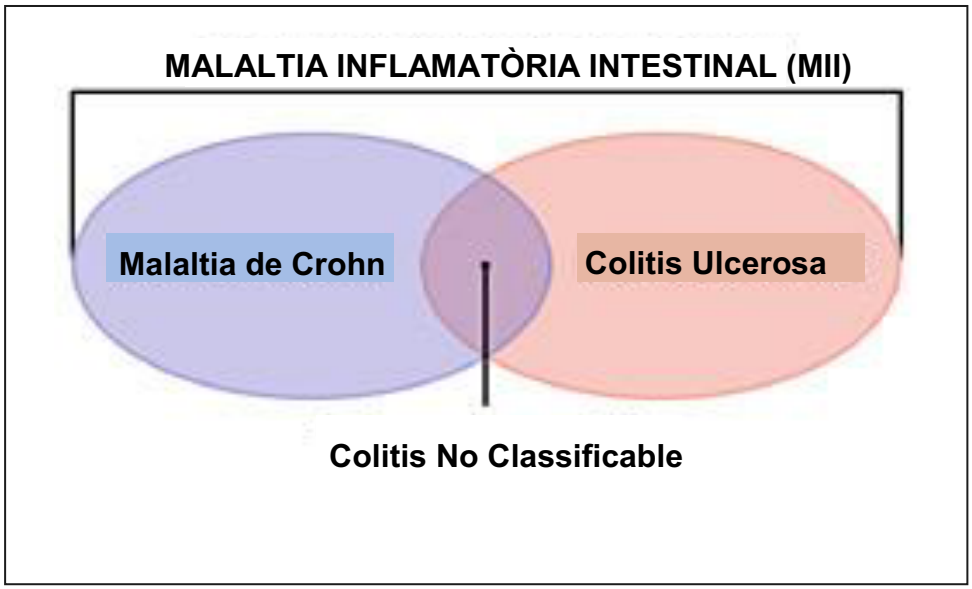


Figura 1. Malaltia Inflamatòria Intestinal

En el curs clínic de la malaltia, la cronicitat consisteix en l'alternança de períodes d'inactivitat, fases de remissió, amb períodes d'activitat clínica de diferent intensitat, brots o recidives. La CU és una inflamació de la mucosa del colon que afecta al recte en el 95% dels casos i s'extén de manera proximal i contínua en una longitud variable, pot afectar tot el colon (Figura 2).

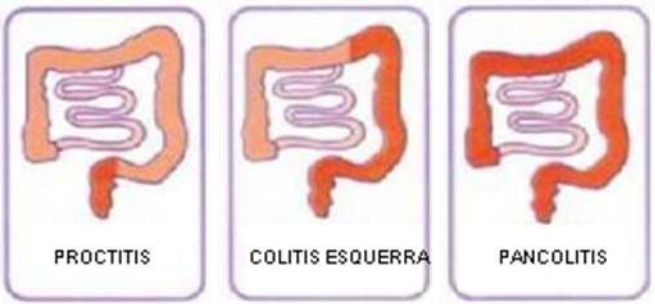


Figura 2. Zones d'afectació de la Colitis Ulcerosa. Adaptat de http://www.accurioja.com/colitis.html

La MC pot afectar qualsevol zona del tracte digestiu, encara que de manera predominant apareix en el segment intestinal que envolta la vàlvula ileo-cecal o en l'intestí gruixut (Figura 3). Sol afectar diferents segments del tracte gastro-intestinal entre els que hi ha zones histològicament normals. A diferència de la CU en què hi ha afectació localitzada a la lamina propria, en la MC l'afectació de l'intestí és transmural i per tant afecta totes les capes de l'intestí.

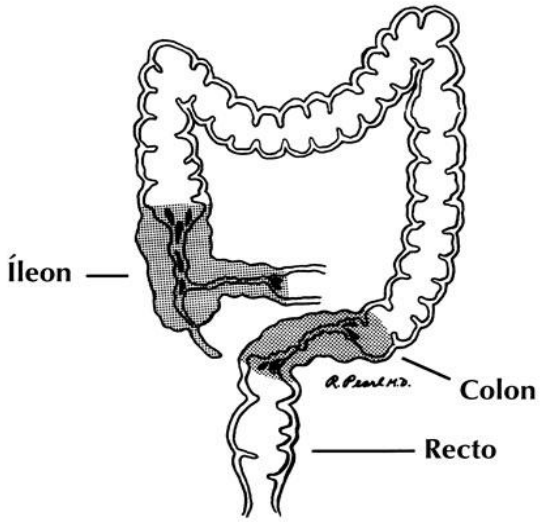


Figura 3. Zones de més afectació en la Malaltia de Crohn.

http://www.fascrs.org/patients/conditions/spanish_brochures/enfermedad_de_crohn/

2) MALALTIA DE CROHN

És una malaltia amb una incidència d'antre 1 i 10 casos cada 100.000 habitants. El diagnòstic de la malaltia és molt rar abans dels 10 anys d'edat i té pics d'incidència màxima al voltant de la 2a i 3a dècada de vida. La distribució per sexes és similar[1]. La inflamació intestinal apareix com a conseqüència d'una resposta immunològica anormal a components de la llum intestinal en individus genèticament predisposats. Al ser una malaltia d'afectació transmural, la inflamació pot localitzar-se a tot el gruix de la paret intestinal. Hi ha afectació de la mucosa, la submucosa, la *muscularis propia*, la subserosa i el greix mesentèric (Figura 4).

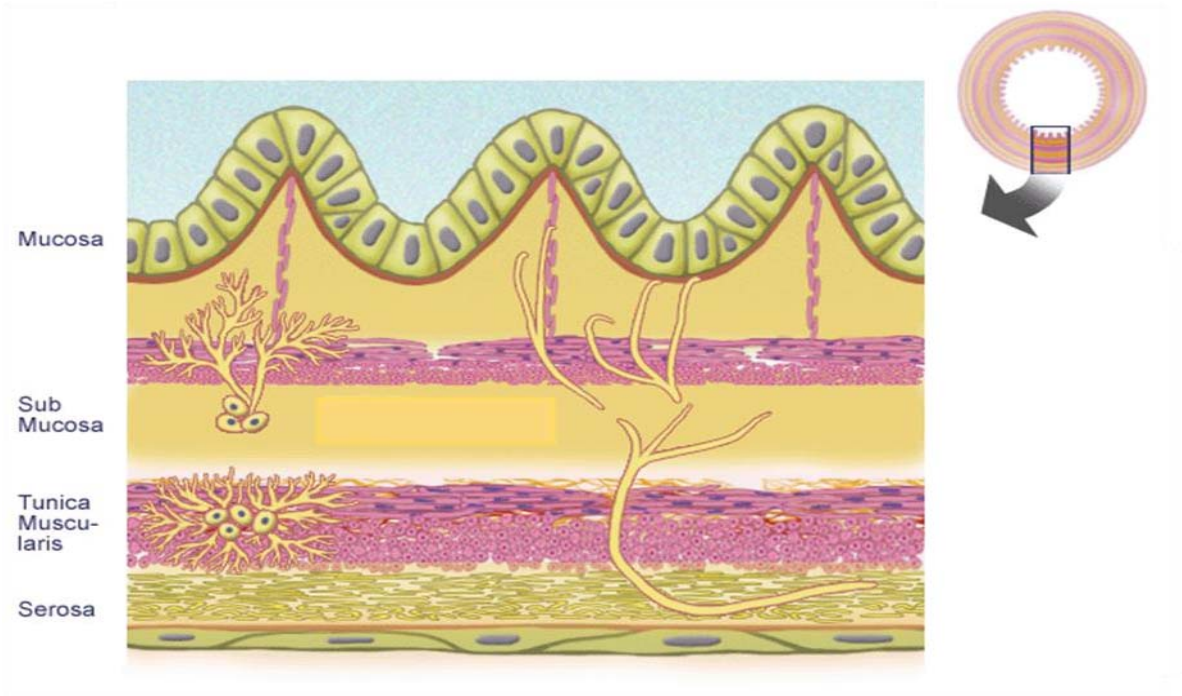


Figura 4. Estructura de la paret intestinal

http://alexandria.healthlibrary.ca/documents/notes/bom/unit_4/unit%204%202005/Images%202005/lec%2015-%20fig%201.gif

El comportament clínic de la malaltia es presenta en diferents patrons, aquests patrons no són estables sinó que al llarg del temps es poden presentar tots ells en un mateix pacient i són[2] (Figura 5):

- Patró **inflamatori**: presència d'úlceres superficials, que es poden convertir en més profundes, i inflamació.
- Patró **fibro-estenosant**: la estenosi es defineix com un estretament de la llum intestinal degut al procés inflamatori de llarga evolució, mentre que la fibrosi resulta d'un procés anòmal de reparació del teixit i es caracteritza per la presència d'un teixit cicatricial. Es caracteritza per una pobra resposta al tractament mèdic i requereix un procés quirúrgic.
- Patró perforant o **fistulitzant**: la inflamació en l'ili pot evolucionar cap a unes estructures transmurals, les fístules, que poden causar perforació de l'intestí o comunicacions amb altres òrgans.

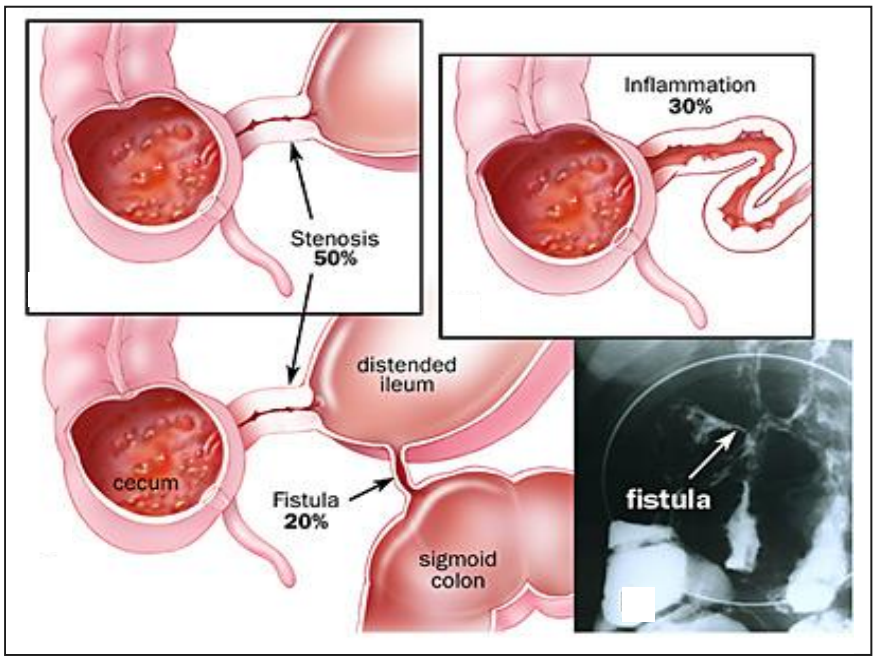


Figura 5. Comportament clínic de la Malaltia de Crohn.

http://www.hopkins-gi.org/GDL_DiseaseLibrary.aspx?SS=&CurrentUDV=31

En els pacients amb un patró fibro-estenosant es dóna una reducció de la llum que dificulta el trànsit intestinal. Aquest fet condiciona l'aparició de dolor abdominal amb distensió abdominal, nàusees i vòmits, molts cops en absència de símptomes i paràmetres analítics suggestius d'activitat inflamatòria. El desenvolupament d'un fenotip fibròtic doncs, condiciona de manera molt important la qualitat de vida d'aquests pacients, ja que, a més dels símptomes mencionats, és causa de repetits ingressos hospitalaris deguts a episodis d'oclusió intestinal.

En el moment del diagnòstic tan sols el 10% dels pacients amb MC presenten un patró fibro-estenosant, en el transcurs de la malaltia el desenvolupament de fibrosi és freqüent i pot afectar al 32% dels pacients amb MC[3]. Es creu que el desenvolupament de fibrosi intestinal és conseqüència d'un procés d'inflamació crònica i una reparació anòmala del teixit afectat, això explicaria perquè alguns pacients inicialment diagnosticats amb un patró inflamatori de la malaltia desenvolupen un patró fibro-estenosant en un període de 10 anys. Cal tenir en compte però que a pesar dels avenços terapèutics en el tractament de la MC la incidència del patró fibro-estenosant no ha canviat significativament, per tant el control de la inflamació a nivell clínic no sembla que tingui cap efecte en el procés fibrogènic.

A dia d'avui no hi ha disponible cap tractament mèdic específic per a aquesta complicació i en molts casos l'únic tractament possible és la resecció quirúrgica del segment intestinal afectat. Aquesta opció, a més,

presenta l'inconvenient de no ser una solució definitiva ja que la malaltia reapareix en un gran nombre d'aquests pacients, pocs anys després de la primera intervenció i un 40% d'aquests pacients requereixen una segona intervenció quirúrgica.

3) FIBROSI

Els òrgans afectats per un procés inflamatori responen previsiblement intentant guarir el dany tissular provocat pels mediadors de la inflamació. Si la inflamació ha estat transitòria i moderada el restabliment de l'arquitectura normal del teixit és complert. En els processos que cursen amb inflamació crònica, com la MC, la severitat i l'abast de la lesió pot excedir la capacitat regenerativa del teixit afectat, el qual es defensa desenvolupant una resposta fibrogènica que resulta en la formació de teixit cicatricial, un procés conegut com a fibrosi. Aquest teixit cicatricial fa que l'òrgan funcioni anormalment.

3.1. Factors genètics associats al desenvolupament del patró fibro-estenosant en la MC

L'any 1996 es va identificar per primer cop un locus de susceptibilitat per la MC localitzat a prop de la regió centromèrica del cromosoma 16, concretament en 16q21[4,5]. Mitjançant anàlisis posteriors d'aquesta regió es va trobar una forta associació amb el gen NOD2 (de l'anglès *nucleotide-binding oligomerization domain containing 2*) també conegut com a CARD15 (de l'anglès *caspase recruitment domain family, member 15*). Aquest gen forma part de la superfamília de reguladors de l'apoptosi Apaf-1/CED-4 (de l'anglès *apoptotic protease activating factor 1* i *cell death protein 4*). La proteïna és d'expressió intra-cel·lular en monòcits i macròfags, cèl·lules en les que sembla que funciona com a sensor de productes bacterians. S'ha demostrat expressió d'aquesta proteïna també en fibroblasts intestinals[6], un tipus cel·lular amb un paper clau en el desenvolupament de la fibrosi intestinal.

S'han descrit tres polimorfismes en NOD2 implicats en MC i que condicionen un patró fibro-estenosant de la malaltia[7]. Es coneixen com a SNP8, SNP12 i SNP13 i corresponen a les mutacions puntuals 2104C>T, 2722G>C i 3020insC que resulta en un codó stop prematur i una proteïna de 1007 aminoàcids enlloc de 1040. En població caucàsica no jueva s'ha demostrat que aquestes variants tenen un *odds ratio* de 2,2 (95% CI: 1.84-2,62), 2,99 (95% CI: 2,38-3,74) i 4,09 (95% CI: 3,23-5,18) respectivament per a la presentació de la malaltia. Per a portadors de dos al·lels, la *odds ratio* és de 17,1 (95% CI: 10,7-27,2). A

més, aquestes variants condicionen un increment moderat en el risc de presentar un patró fibro-estenosant amb una *odds ratio* de 1,94 (95% CI: 1,61-2,34)[8].

Més recentment, s'ha implicat un altre gen en el desenvolupament de la MC. És el receptor de fractalquina CXC3R1 (de l'anglès *chemokine (C-X-C motif) 3 receptor 1*), concretament s'han descrit dos polimorfismes en el gen d'aquest receptor de citocina, 249I i 280M, associats al patró fibro-estenosant en la MC[9,10]. Aquests polimorfismes són funcionalment rellevants ja que disminueixen l'afinitat lligam-receptor[11,12]. Una de les funcions de la fractalquina és inhibir l'expressió de TIMP-1 (de l'anglès, Tissue Inhibitor of Metalloproteinases 1), una proteïna que com veurem més endavant té un paper en el desenvolupament de la fibrosi en diferents teixits. Els polimorfismes en el receptor de fractalquina mencionats més amunt dificulten la unió de la fractalquina al receptor conduint a una major producció de TIMP-1[13], fet que podria explicar l'associació entre aquests polimorfismes i el patró fibro-estenosant en la MC.

3.2. Síntesi de col·lagen

El desenvolupament de fibrosi és el resultat d'un desequilibri entre la deposició de matriu extra-cel·lular i la seva degradació. L'increment en la producció de col·lagen i altres components de la matriu extra-cel·lular (MEC) pot venir donat quan les cèl·lules productores, fibroblasts i miofibroblasts, en produeixen més quantitat o bé hi ha un major nombre de cèl·lules productores o bé per una combinació dels dos factors. En la fibrosi tissular incrementa el nombre de cèl·lules productores de matriu extracel·lular, aquest increment és secundari a una major proliferació d'aquestes cèl·lules i a una disminució en la mort cel·lular programada.

En la paret intestinal afecta de pacients amb MC fibro-estenosant s'ha comprovat la presència de nivells elevats de mARN i proteïna pels col·làgens tipus I, III, IV i V[14]. La citocina TGF-β (de l'anglès *transforming growth factor-β*) té un paper molt important en el desenvolupament de fibrosi i especialment en la regulació de l'expressió de proteïnes de la matriu extracel·lular a través de la senyalització per Smad3. És interessant destacar que la regió promotora dels gens COL1A1, COL1A2, COL3A1, COL5A2, COL6A1 i COL6A3, tots ells codifiquen per diferents tipus de col·lagen, conté elements d'unió a Smad3[15].

S'ha vist que els fibroblasts intestinals aïllats de zones fibro-estenòtiques tenen una expressió de TGF-β1 molt elevada i com a conseqüència d'això les àrees de l'intestí afectades de fibrosi contenen alts nivells

d'aquesta citocina[16]. A més, també s'ha demostrat la sobre expressió dels receptors per TGF-β en l'intestí de pacients amb MC[17].

S'ha proposat el següent model sobre el paper del TGF-β en el desenvolupament de fibrosi intestinal. Quan hi ha un dany en el teixit es dóna la degranulació de plaquetes. Les plaquetes alliberen grans quantitats de TGF-β, aquest actua com a factor quimiotàctic per macròfags, monòcits i fibroblasts. Un cop al teixit els monòcits també alliberen TGF-β que actua sobre els fibroblasts i acaba conduint a la producció de col·làgens i altres proteïnes de la matriu extra-cel·lular[18] (Figura 6).

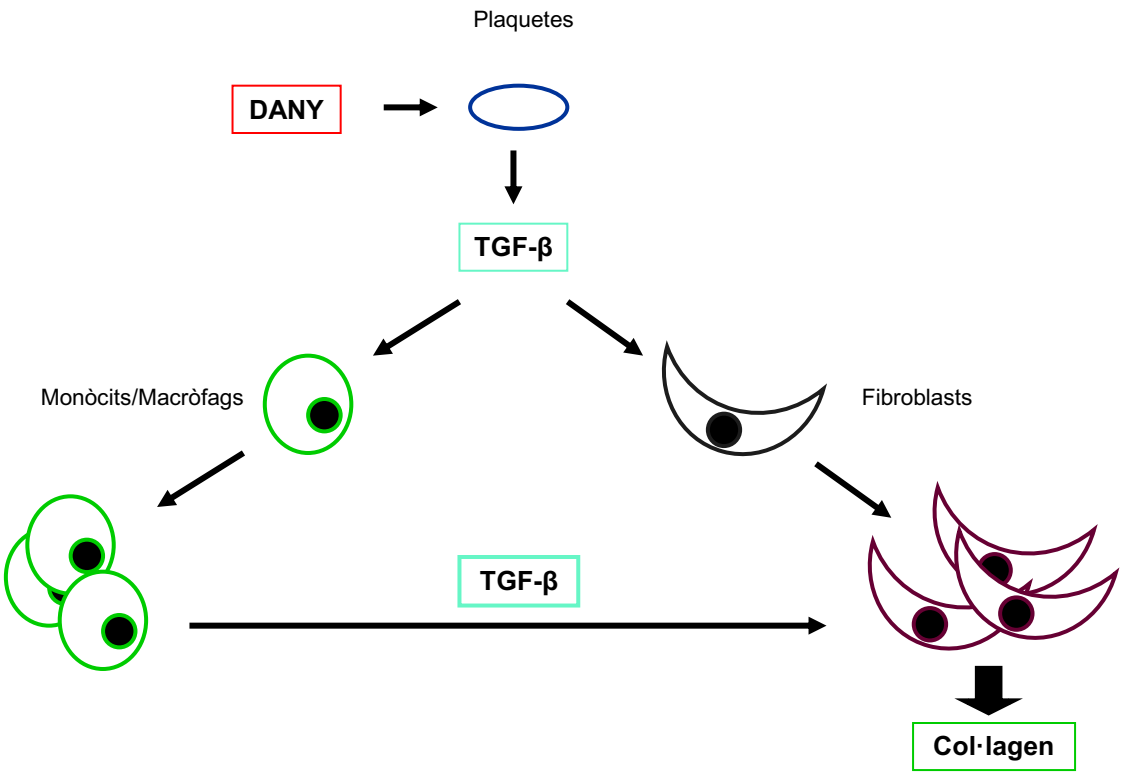


Figura 6. Paper del TGF-β en la fibrosi intestinal.

El TGF-β es troba en l'espai inter-cel·lular en forma inactiva unit al complex LAP-LTBP (de l'anglès *Latency-Associated Peptide – Latent TGF-β-binding protein*). Aquest associació impedeix la unió de TGF-β al seu receptor. L'activació del TGF-β requereix el seu alliberament del complex LAP-LTBP procés que pot ser mediat per vàries proteases com la plasmina[19], MMP-2 i MMP-9[20] (de l'anglès *matrix metalloproteinases 2* i *9*) o bé Tsp-1[21] (de l'anglès *thrombospondin 1*).

La unió de TGF-β al seu receptor indueix la fosforilació de Smad2 i Smad3, aquestes un cop fosforilades s'uneixen a Smad4 i transloquen al nucli on estimulen l'expressió de gens de matriu extra-cel·lular. La

senyalització per Smad3 està regulada negativament mitjançant les Smads inhibitòries, Smad6 i Smad7, que competeixen amb Smad3 per la unió al receptor I de TGF-β[22] (Figura 7).

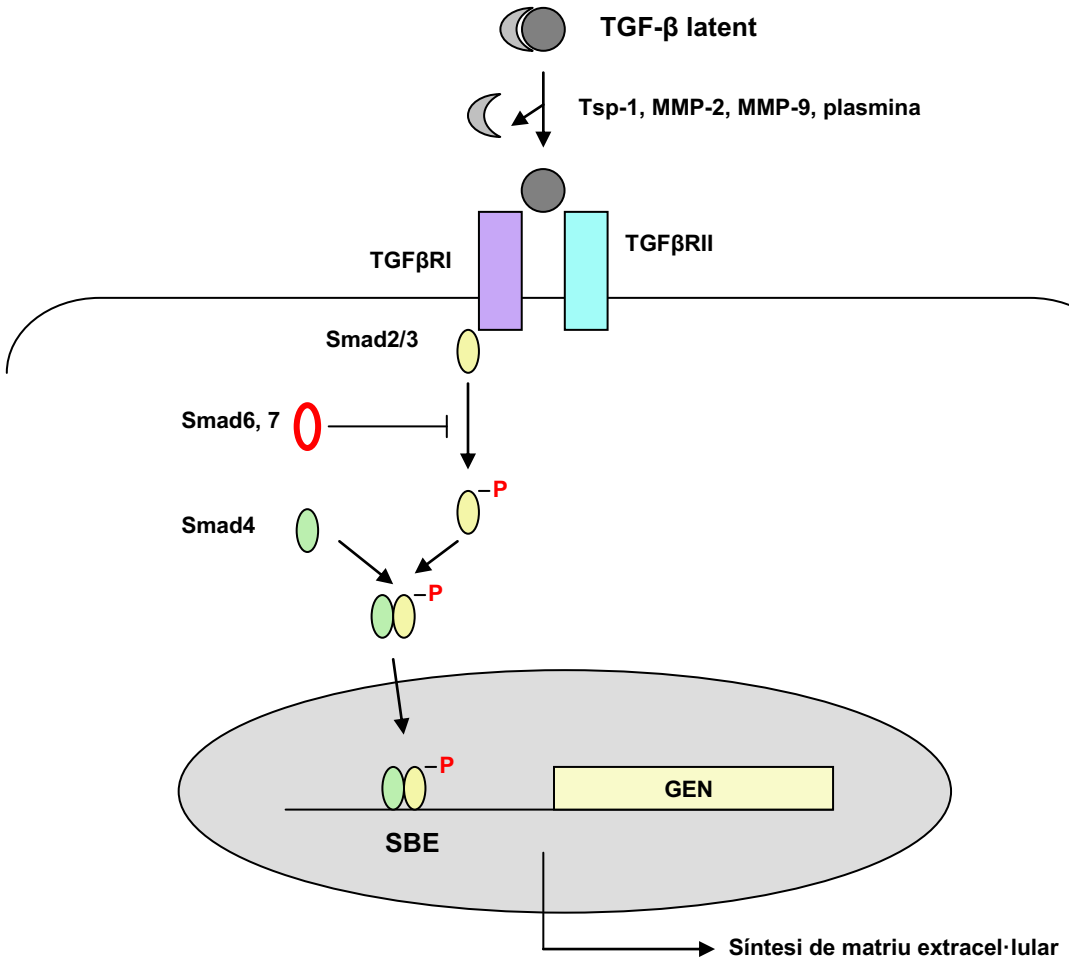


Figura 7. Via de senyalització del TGF-β. Adaptat de Leask 2004.

3.3. Metaloproteinases i degradació de la matriu extracel·lular

El paper del TGF-β en la fibrosi no es limita a l'activació de la síntesi de MEC sinó que a més, inhibeix la degradació de la matriu mitjançant la regulació negativa en l'expressió de MMPs, els enzims responsables de la degradació de MEC, i la regulació positiva en l'expressió dels inhibidors de les MMPs, els TIMPs.

Alguns estudis suggereixen que l'efecte del TGF-β en la regulació del sistema MMP-TIMP també podria ser depenent de la via d'Smad3. Així s'ha vist que en fibroblasts aïllats de pell, el TGF-β regula negativament l'expressió de MMP-1 i que aquesta regulació està mediada per Smad3[23] i que la inducció en l'expressió de TIMP-1 també és depenent d'Smad3[24]. En aquest sentit, DiSabatino i cols. van demostrar l'any 2009 major presència de TGF-β, Smad3 fosforilada i nivells més elevats de TIMP-1 en

àrees de fibro-estenosi intestinal en pacients amb MC. A més, van demostrar que aquestes zones afectades de fibro-estenosi tenen una menor expressió de Smad7, MMP-12 i MMP-3[25].

En els teixits, de manera fisiològica, hi ha un equilibri entre la síntesi i la degradació de col·lagen i altres components de la matriu extra-cel·lular. Les metaloproteinases són un nombrós grup d'enzims implicats en aquest procés. Les MMPs són una subfamília d'enzims dependents de zinc i calci. En l'espècie humana s'han descrit 23 MMPs diferents. Totes elles contenen un pèptid senyal a l'extrem N-terminal que dirigeix l'enzim cap a la via secretora, un pro-domini que confereix latència a l'enzim i un domini catalític amb unió a zinc. La majoria de MMPs són secretades com a pro-enzims i la seva activació té lloc en l'espai extra-cel·lular mitjançant una reacció proteolítica[26].

En el seu conjunt les MMPs tenen la capacitat de degradar la majoria de proteïnes de la matriu extra-cel·lular[27]. A més, també poden processar un gran nombre d'altres proteïnes com factors de creixement, citocines, quimiocines, receptors i altres MMPs[28]. L'expressió de la majoria de MMPs en teixits normals és baixa i la inducció es dóna quan és necessària una remodelació de la matriu extra-cel·lular (MEC).

S'ha demostrat que TNF-α (de l'anglès *tumor necrosis factor-α*) estimula l'expressió de MMP-1 en fibroblasts[29]. En el nostre grup hem pogut observar que TNF-α també té un paper en la inducció de MMP-3 en fibroblasts intestinals humans (FIH).

L'acció de les MMPs és inhibida pels inhibidors tissulars de metaloproteinases, TIMPs. Els TIMPs interaccionen amb el domini actiu de les MMPs bloquejant la seva activitat[27]. El paper dels TIMPs en el desenvolupament de la fibrosi podria ser doble. Per una banda inhibeixen l'acció de les MMPs i per tant inhibeixen la degradació de MEC i la conseqüent acumulació de fibra. D'altra banda TIMP-1 podria regular la divisió cel·lular i l'apoptosi. S'ha demostrat que TIMP-1 suprimeix l'apoptosi de cèl·lules estrellades hepàtiques, responsables de la fibrosi hepàtica, a nivell *in vitro* i *in vivo*[30]. És interessant destacar que el paper de TIMP-1 en la inhibició de l'apoptosi de cèl·lules estrellades hepàtiques és mediat via MMPs. Intentant explicar aquesta troballa els autors formulen dues hipòtesis, d'una banda expliquen que la MEC conté múltiples llocs d'unió per a citocines pro-apoptòtiques que serien alliberades gràcies a l'acció de les MMPs, a l'estar aquestes inhibides per TIMP-1 no es donaria l'alliberament d'aquestes citocines protegint la vida cel·lular. D'altra banda, hipotetitzen que una MEC intacta proporciona a les cèl·lules senyals de supervivència cel·lular ja que la MEC proveeix aquestes cèl·lules amb llocs d'unió on les cèl·lules poden adherir-se i proliferar.

En aquest sentit cobra importància una proteïna present en la MEC anomenada fibronectina (FN). La FN promou la supervivència de fibroblasts[31,32], la proliferació[33] i la migració a través d'una xarxa tridimensional de MEC[34]. Aquestes accions requereixen la unió dels receptors d'integrina en la membrana dels fibroblasts a la xarxa de FN a través de la seqüència Arg-Gly-Asp en la desena repetició de FN tipus III[35]. Recentment, s'ha demostrat que la FN conté tres llocs d'unió al factor de creixement derivat de plaquetes-BB (PDGF-BB, de l'anglès *platelet derived growth factor-BB*) que és un potent factor mitogènic per a fibroblasts i és important per la seva supervivència[36]. Basant-se en les seves troballes, els autors proposen un model per explicar el paper de la FN en la protecció davant l'apoptosi dels fibroblasts. En aquest model la integrina α5β1 de la membrana del fibroblast s'uniria als dominis centrals de la FN. Aquesta unió estaria flanquejada per receptors de PDGF i altres receptors de factors de creixement, els quals estarien units als seus respectius lligams i alhora units a la molècula de fibronectina (Figura 8).

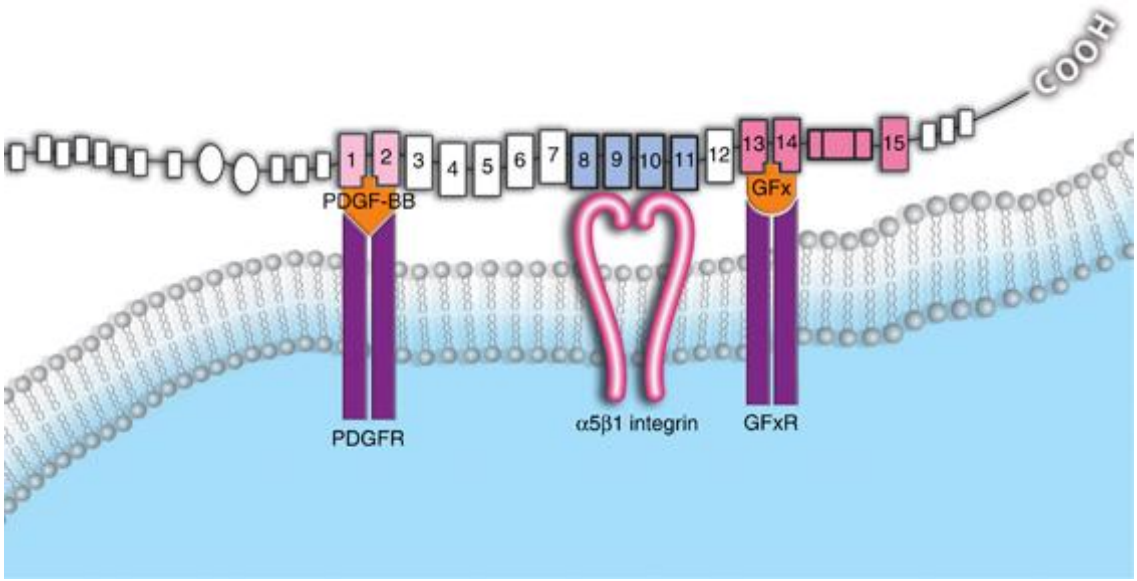


Figura 8. Paper de la fibronectina en la supervivència de fibroblasts.

http://www.nature.com/jid/journal/v131/n1/fig_tab/jid2010253f9.html#figure-title

3.4. Mediadors cel·lulars de la fibrosi

És ben sabut que diferents tipus de cèl·lules mesenquimals, com els fibroblasts, els miofibroblasts i les cèl·lules musculars llises, juguen un paper fonamental en el desenvolupament de la fibrosi en diferents teixits. En l'intestí inflamat, les cèl·lules mesenquimals locals es diferencien i des diferencien entre els tres fenotips cel·lulars. Aquests fenotips estan caracteritzats per la presència o absència de actina de múscul llis α (α-SMA, de l'anglès *smooth muscle actin-α*), vimentina i desmina (Taula 2).

TAULA 2. Fenotips cèl·lules mesenquimals

CÈL·LULA MESENQUIMAL	α-SMA	VIMENTINA	DESMINA
FIBROBLAST	NO	SI	NO
MIOFIBROBLAST	SI	SI	NO
C. MUSCULAR LLISA	SI	NO	SÍ

Quan els fibroblasts intestinals són exposats a factor de creixement similar a insulina I (IGF-I, de l'anglès *insuline like growth factor I*), factor de creixement bàsic de fibroblasts (bFGF, de l'anglès *basic fibroblast growth factor*), PDGF i a les citocines pro inflamatòries interleucina-1β (IL-1β) i TNF-α, aquestes cèl·lules esdevenen activades i es multipliquen[37].

Altres molècules presents en la MC com són la FN, PDGF, IGF-I i TGF-β1 actuen com a factors quimiotàctics per a fibroblasts[38,39] provocant l'acumulació de fibroblasts activats en el lloc de la lesió.

Altres cèl·lules importants en el desenvolupament de la fibrosi en diferents òrgans són les cèl·lules estrellades (SC, de l'anglès *stellate cells*). Aquestes cèl·lules són precursors de cèl·lules mesenquimals i contribueixen a la fibrosi, en particular a la fibrosi hepàtica i pancreàtica, ja que tenen la capacitat de diferenciar-se a fibroblasts activats[40,41]. Existeix poca informació sobre aquestes cèl·lules en l'intestí encara que s'ha vist que cèl·lules estrellades derivades de mucosa afecta de pacients amb MII es diferencien a fibroblasts activats i produeixen col·lagen en major mesura que cèl·lules aïllades de controls[42].

Una font important de fibroblasts són els fibròcits, perícits i cèl·lules epitelials (Figura 9).

Els **fibròcits** són progenitors mesenquimals circulants derivats de medul·la òssia. Aquestes cèl·lules produeixen col·lagen tipus I i es diferencien a fibroblasts *in vitro* i *in vivo*[43,44]. S'ha vist que els fibròcits contribueixen a la població de fibroblasts en diferents patologies[45-47] encara que es desconeix la seva contribució a la MII.

Els **perícits** són cèl·lules d'origen mesenquimal que en el teixit se situen properes a capil·lars i petits vasos sanguinis on controlen l'angiogènesi[48]. Els perícits també es poden diferenciar a fibroblasts i podrien contribuir a la fibrosi intestinal encara que no existeixen estudis en aquest sentit.

Un fenomen que està cobrant protagonisme en els últims anys és la transició epiteli-mesenquima (TEM). Es caracteritza per una transformació dramàtica en el fenotip i la funció cel·lular. En el procés de TEM, les **cèl·lules epitelials** adopten una morfologia en agulla, perden els marcadors de cèl·lula epitelial, guanyen marcadors típics de fibroblast i desenvolupen la capacitat de produir col·lagen i fibronectina[49]. Aquest fenomen ha estat implicat en el desenvolupament de fibrosi en diferents òrgans[50,51].

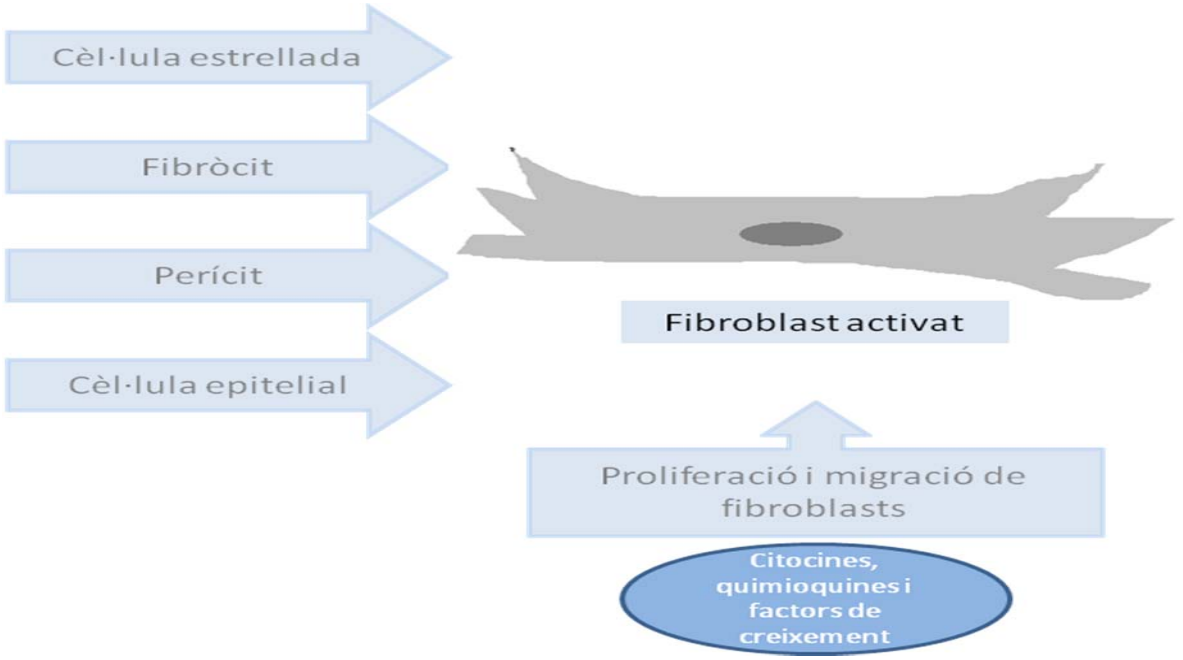


Figura 9. Cèl·lules precursores de fibroblasts.

3.5. Models experimentals de fibrosi

Existeixen pocs models experimentals específicament dissenyats per a reproduir la fibrosi intestinal. Aquest fet ha limitat la recerca i desenvolupament de noves estratègies de tractament anti-fibrogènic a nivell intestinal. L'any 2005, Vallance i cols. van descriure un model basat en la transfecció d'un vector adenoviral que contenia TGF-β1 d'activació espontània administrat en forma d'ènema. Els autors van observar que la transfecció provocava l'aparició de fibroblasts activats i una major producció de col·lagen a nivell intestinal que va causar obstrucció intestinal[52]. Aquest treball no només proporciona un nou model experimental de fibrosi intestinal sinó que, a més, demostra el paper clau del TGF-β en la fibrogènesi.

Anteriorment a aquest treball, Lawrance i cols. van descriure un model de fibrosi intestinal en ratolí. En aquest cas, la fibrosi es va induir mitjançant l'administració intra-colònica repetida i a dosis creixents de l'haptè TNBS (de l'anglès *trinitrobenzene sulfonic acid*). Aquest model consistia en la modificació d'un model molt utilitzat durant anys en l'estudi dels processos inflamatoris que es donen en la MII i que es basa en l'administració d'una dosi alta de TNBS[53]. Mitjançant l'administració repetida de dosis més baixes de TNBS els autors van aconseguir instaurar en el ratolí un grau constant i important de fibrosi intestinal[54] (Figura 10).

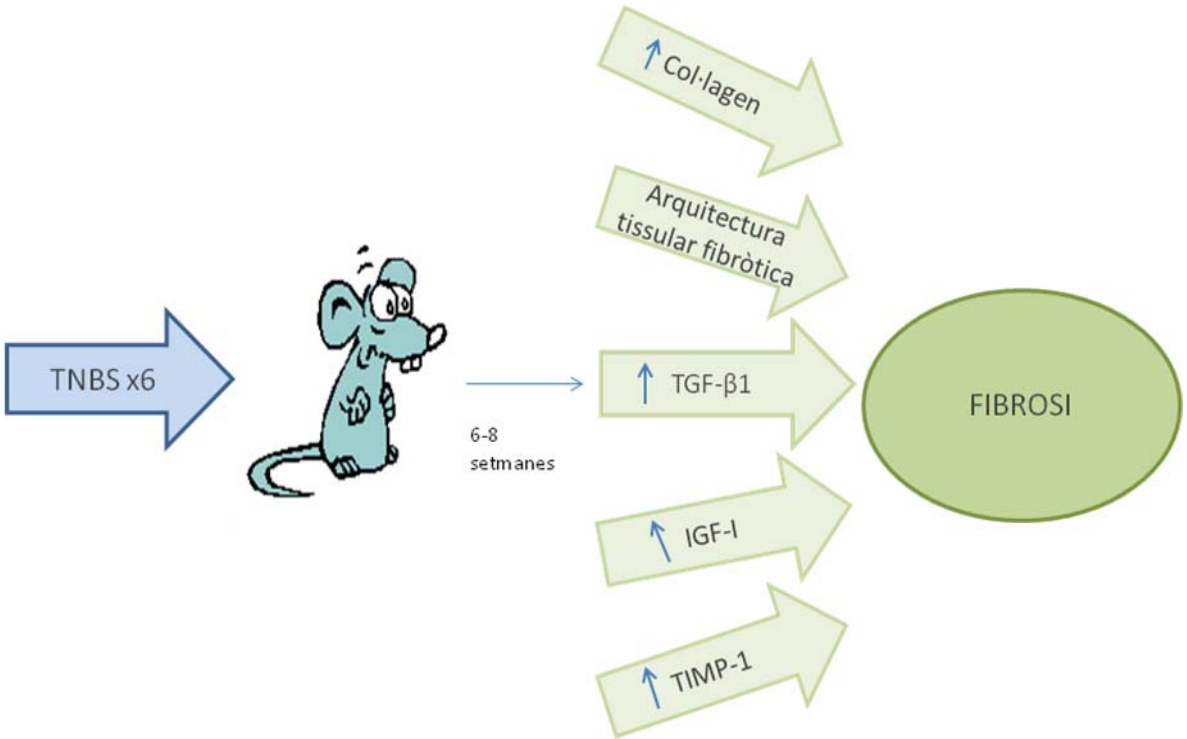


Figura 10. Model de fibrosi per TNBS en ratolí.

El model clàssic d'administració intra-colònica d'una dosi de TNBS és capaç de desenvolupar un cert grau de fibrosi intestinal en la rata tal com demostren alguns estudis[55,56].

3.6. Mecanismes de resolució de la fibrosi

Durant molts anys, la fibrosi s'havia considerat un procés irreversible. Diversos estudis centrats en el procés de fibrosi hepàtica demostren que podria no ser així. L'administració intra peritoneal de CCl_4 durant quatre setmanes és capaç d'induir fibrosi hepàtica en la rata. Aquest tractament provoca un augment en la producció i deposició de fibronectina, col·lagen I, col·lagen IV i laminina[57] i un augment en el nombre de cèl·lules amb una morfologia típica de fibroblasts[58] en el fetge dels animals tractats.

L'any 1998 Iredale i cols.[58] van analitzar animals tractats amb CCl_4 als 3, 7 i 28 dies d'haver acabat el tractament. Van observar que després de 28 dies sense rebre tractament amb CCl_4 el fetge de la rata tenia uns nivells de col·lagen i una histologia molt semblants als animals control, no tractats amb CCl_4. Van demostrar que la transformació del teixit fibròtic a teixit normal era deguda a l'apoptosi de cèl·lules estrellades hepàtiques (HSC de l'anglès *hepatic stellate cells*) alhora que veien una ràpida disminució en l'expressió de TIMP-1 i TIMP-2, mentre que l'activitat de la MMP-13, coneguda també com a col·lagenasa, es mantenia constant. Els autors conclouen que l'apoptosi de les HSC és el pas clau per a la resolució de la fibrosi hepàtica en aquest model encara que aquest fet per si sol no és suficient ja que cal la degradació de la MEC acumulada, això, en aquest estudi, van demostrar que era degut a la inhibició en l'expressió de TIMP-1 i TIMP-2 sense afectar l'activitat de MMP-13.

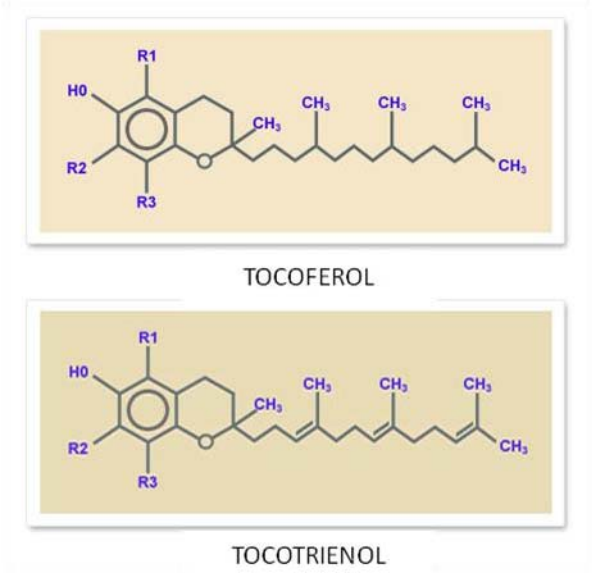
Temps més tard, es va confirmar que l'apoptosi de HSC és un pas clau en la resolució de la fibrosi hepàtica. Wright i cols. van demostrar que la gliotoxina és capaç d'induir apoptosi de HSC *in vitro* a baixes concentracions i que la injecció de gliotoxina en rates tractades amb CCl_4 accelera la recuperació de la fibrosi hepàtica[59].

El factor de creixement d'hepatòcits (HGF de l'anglès *hepatocyte growth factor*) redueix la fibrosi pulmonar en models murins[60,61]. Se sap que HGF és un potent inductor de les MMPs[62] i que aquestes indueixen l'apoptosi de fibroblasts activats mitjançant la degradació de fibronectina[63].

Així doncs la inducció d'apoptosi de les cèl·lules responsables de la fibrosi en diferents òrgans sembla ser un mecanisme clau en la resolució de la fibrosi. En aquest sentit, els tocotrienols han atret l'atenció dels investigadors degut al seu potencial anti-fibrogènic.

4) FRACCIÓ RICA EN TOCOTRIENOLS

La vitamina E és un fito-nutrient molt important present en olis comestibles. El terme vitamina E agrupa 8 isòmers, 4 tocoferols (alfa, beta, gamma i delta) i 4 tocotrienols (alfa, beta, gamma, delta). La diferència entre tocoferols i tocotrienols és que aquests últims tenen tres dobles enllaços en la cadena lateral mentre que els tocoferols no en tenen cap. La diferència entre les isoformes alfa, beta, gamma i delta és la presència o no de grups metil en les posicions R1, R2 i R3, tal com es detalla en la figura 11.



	R1	R2	R3
alfa	CH3	CH3	CH3
beta	CH3	H	CH3
gamma	H	CH3	CH3
delta	H	H	CH3

Figura 11. Composició de la Vitamina E.

Adaptat de: http://www.tocotrienol.org/index.php?option=com_content&view=article&id=84&Itemid=75

Els olis comestibles que s'originen a partir de plantes són font de tocotrienols. En concret, l'oli que s'origina a partir del fruit de la palma és especialment ric en aquests compostos. La fracció rica en tocotrienols (FRT) conté una barreja de tocotrienols i tocoferol extrets i concentrats a partir de l'oli de palma mitjançant un procés patentat per Carotech (Figura 12). També conté altres fito-nutrients com fito-esterols, coenzim Q10 i una barreja de carotenoides.

	TOCOFEROLS					TOCOTRIENOLS			
	ALFA	BETA	GAMMA	DELTA		ALFA	BETA	GAMMA	DELTA
TRF*	152	-	-	-		205	-	439	94

* Miligrams per 1000 grams

Figura 12. Composició de la Fracció Rica en Tocotrienols.

La proteïna transportadora de tocoferol (TTP de l'anglès *tocopherol transport protein*), és una proteïna soluble de 32 kDa d'expressió hepàtica que uneix i transporta selectivament α-tocoferol[64]. Això ha fet que la investigació en tocotrienols hagi quedat a l'ombra de la investigació duta a terme amb els tocoferols, principalment l'α-tocoferol. De fet, la investigació amb tocotrienols representa l'1% de tota la investigació en vitamina E publicada a PubMed[65]. Tot i la controvèrsia en referència a la bio-disponibilitat dels tocotrienols de la dieta, els coneixements actuals han fet canviar radicalment la visió de què els tocotrienols no són absorbibles. Així s'ha vist que els tocotrienols de la dieta s'absorbeixen, arriben a nivells mesurables en plasma[66] i arriben a distribuir-se en teixits com el cervell, teixit adipós, pell o glàndules mamàries[67-70].

Els tocotrienols han atret l'atenció dels investigadors degut a què han demostrat tenir múltiples propietats beneficioses per la salut. L'evidència acumulada ens suggereix que els tocotrienols són bons agents antineoplàstics, neuro- i cardio-protectors i poden disminuir els nivells de colesterol[71,65].

Els isoprenoides, com els tocotrienols però no els tocoferols, són molècules amb un gran potencial antitumoral ja que inhibeixen el creixement i indueixen l'apoptosi *in vitro* de cèl·lules canceroses[72]. Concretament, les isoformes γ- i δ-tocotrienol són les més potents en la inducció d'apoptosi de cèl·lules tumorals.

L'any 2007, Rickmann i cols.[73] van demostrar que aquests compostos, la FRT, també tenien efectes anti-proliferatius i pro-apoptòtics, no només en cèl·lules tumorals sinó també en cèl·lules estrellades pancreàtiques (PSCs, de l'anglès *pancreatic stellate cells*). Les PSCs són cèl·lules d'origen mesenquimal similars als fibroblasts que han estat identificades com a les responsables de l'expansió de la matriu extracel·lular en la fibrogènesi pancreàtica.

D'aquest estudi és interessant destacar que els efectes de la FRT sobre PSCs són deguts a les isoformes β-, γ- i δ-tocotrienols. A més, la FRT té efectes anti-proliferatius, pro-apoptòtics i pro-autofàgics sobre cèl·lules activades però no sobre cèl·lules quiescents.

Així doncs aquest estudi obra la porta a una possible teràpia anti-fibrogènica no només en pàncrees sinó també en altres teixits i malalties que cursen amb aquesta manifestació clínica.

OBJECTIUS

HIPÒTESI:

La Fracció Rica en Tocotrienols té un potencial antifibrogènic.

PRIMER OBJECTIU:

Esbrinar els efectes de la fracció rica en tocotrienols sobre la proliferació i l'apoptosi de fibroblasts intestinals humans *in vitro*.

SEGON OBJECTIU:

Estudiar els efectes de la fracció rica en tocotrienols sobre la matriu extracel·lular en fibroblasts intestinals humans *in vitro*.

- Avaluar la producció de proteïnes de matriu extracel·lular.
- Avaluar la producció de proteïnes reguladores de la degradació de la matriu extracel·lular.

TERCER OBJECTIU:

Estudiar la utilitat de la fracció rica en tocotrienols com a tractament antifibrogènic en un model de fibrosi intestinal en la rata.

- Establir i caracteritzar un model de fibrosi intestinal basat en l'administració de TNBS.
- Estudiar l'efecte del pretractament amb fracció rica en tocotrienols en aquest model de fibrosi intestinal

RECULL D'ARTICLES

Articles:

1) Luna J, Masamunt MC, Rickmann M, Mora R, España C, Delgado S, Llach J, Vaquero E, Sans M. Tocotrienols have potent antifibrogenic effects in human intestinal fibroblasts. Inflamm Bowel Dis. 2011;17:732-41. Factor impacte: 4.613. Primer quartil Gastroenterology and Hepatology.

2) Luna J, Masamunt MC, Llach J, Delgado S, Sans M. Palm oil tocotrienol rich fraction reduces extracellular matrix production by inhibiting transforming growth factor-β1 in human intestinal fibroblasts. Clinical Nutrition 2011. Acceptat per publicació. DOI: 10.1016/j.clnu.2011.07.001. Factor impacte: 3.41. Primer quartil Nutrition and Dietetics.

3) Luna J, Mora R, Masamunt MC, Nunes T, Bravo R, Bombí JA, Molero X, Vaquero E, Sans M. Treatment of intestinal fibrosis with tocotrienols in an optimized rat model. Sotmès per a publicació.

1) Primer treball

Tocotrienols have potent antifibrogenic effects in human intestinal fibroblasts.

L'excessiva acumulació de fibroblasts i producció de MEC són fets clau en el desenvolupament de la fibrosi intestinal associada a la MC. Els tocotrienols són components de la Vitamina E que han demostrat posseir efectes antifibrogènics "in vitro" en fibroblasts aïllats del pàncreas de la rata.

L'objectiu d'aquest estudi és investigar els efectes dels tocotrienols sobre la proliferació, apoptosi, autofàgia i síntesi de MEC en fibroblasts intestinals humans.

La FRT redueix la proliferació dels fibroblastes intestinals humans de manera basal i també disminueix la proliferació induïda pel factor bàsic de creixement de fibroblast en aquells fibroblasts aïllats de pacients amb MC i CU però no en fibroblasts control.

La FRT promou l'apoptosi i autofàgia en FIH. L'administració de l'inhibidor de caspases Z-VAD-fmk va bloquejar l'apoptosi però no l'autofàgia, en canvi, l'administració de ciclosporina A va ser eficaç prevenint tant l'apoptosi com l'autofàgia induïda per la FRT, demostrant l'important paper del mitocondri en aquests dos tipus de mort cel·lular.

La FRT disminueix la producció de proteïnes de la MEC, com ara procol·làgen I i laminina γ.

Tocotrienols Have Potent Antifibrogenic Effects in Human Intestinal Fibroblasts

Jeroni Luna, MSc,* Maria Carme Masamunt, MSc,* Mariana Rickmann, PhD,* Rut Mora, MSc,* Carolina España, BSc,* Salvadora Delgado, MD,† Josep Llach, MD,* Eva Vaquero, MD,* and Miquel Sans, MD, PhD*

Background: Excessive fibroblast expansion and extracellular matrix (ECM) deposition are key events for the development of bowel stenosis in Crohn's disease (CD) patients. Tocotrienols are vitamin E compounds with proven in vitro antifibrogenic effects on rat pancreatic fibroblasts. We aimed at investigating the effects of tocotrienols on human intestinal fibroblast (HIF) proliferation, apoptosis, autophagy, and synthesis of ECM.

Methods: HIF isolated from CD, ulcerative colitis (UC), and normal intestine were treated with tocotrienol-rich fraction (TRF) from palm oil. HIF proliferation was quantified by ^{3}H-thymidine incorporation, apoptosis was studied by DNA fragmentation, propidium iodide staining, caspase activation, and poly(ADP-ribose) polymerase cleavage, autophagy was analyzed by quantification of LC3 protein and identification of autophagic vesicles by immunofluorescence and production of ECM components was measured by Western blot.

Results: TRF significantly reduced HIF proliferation and prevented basic fibroblast growth factor-induced proliferation in CD and UC, but not control HIF. TRF enhanced HIF death by promoting apoptosis and autophagy. HIF apoptosis, but not autophagy, was prevented by the pan-caspase inhibitor zVAD-fmk, whereas both types of cell death were prevented when the mitochondrial permeability transition pore was blocked by cyclosporin A, demonstrating a key role of the mitochondria in these processes. TRF diminished procollagen type I and laminin γ production by HIF.

Conclusions: Tocotrienols exert multiple effects on HIF, reducing cell proliferation, enhancing programmed cell death through apoptosis and autophagy, and decreasing ECM production. Considering their in vitro antifibrogenic properties, tocotrienols could be useful to treat or prevent bowel fibrosis in CD patients.

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Key Words: inflammatory bowel disease, fibroblasts, tocotrienol rich fraction

Crohn's disease (CD) is a very heterogeneous condition and more than one-third of CD patients will develop a fibrostenosing phenotype, characterized by progressive narrowing of the intestinal lumen. In these patients abnormal bowel fibrogenesis is due to chronic transmural inflammation and impaired wound healing, which result in massive fibroblast proliferation and an excessive deposition of ECM in the bowel wall. Ultimately, abnormal contraction of the ECM will also contribute to tissue distortion and intestinal obstruction.

Relatively minor progress has been made, to our knowledge, into the molecular mechanisms that lead to bowel fibrosis, as compared to liver, lung, kidney, or skin fibrosis.[1] Several molecules have been shown to be involved in the abnormal bowel fibrogenesis that takes place in stenosing CD patients. Among them, basic fibroblast growth factor (bFGF) and insulin-like growth factor 1 (IGF-1) seem to play a key role in that process. They are upregulated in bowel strictures of CD patients where they promote both fibroblast proliferation and ECM production.[2,3]

More important, no medical treatment for bowel fibrosis has become available to date, in spite of the remarkable success of the new, antiinflammatory therapies recently developed for inflammatory bowel disease (IBD).[4] Due to the lack of medical therapies for bowel fibrosis, most CD patients with a stenosing phenotype will require surgical resection of the involved bowel segment, either once or, often, more times during their lives.

In that regard, a variety of natural dietary constituents, including vitamin E, have recently attracted researcher's attention for their health benefits and harmless consumption profile. In nature, eight substances have been found to have vitamin E activity: a-, b-, c-, and d-tocopherol; and a-, b-, c-, and d-tocotrienol. To date, most efforts have been devoted to a-tocopherol, due to its abundance in the human body and potent antioxidant activity.[5] However, dietary tocotrienols are well absorbed, easily distributed throughout the body tissues, and could provide greater

health benefit than a-tocopherol, due to their antiproliferative, neuroprotective, and cholesterol-lowering properties.[5] The suppressive effects of tocotrienols on tumor growth are attributed to their ability to induce both cell cycle arrest and apoptosis in transformed cells.[6–9] On the contrary, a-tocopherol is not effective in inducing apoptosis in cancer cells.[9]

A previous study from our group demonstrated that tocotrienols can induce apoptosis and autophagy in rat pancreatic fibroblasts in vitro.[10] The purpose of the present study was to characterize the effect of tocotrienols in human intestinal fibroblast (HIF) obtained from CD patients in order to ascertain whether tocotrienol-rich fraction (TRF) has an antifibrogenic effect on intestinal fibroblasts and could constitute a potential therapeutic approach for bowel fibrosis in CD patients.

MATERIALS AND METHODS

Reagents and Antibodies

Cell culture flasks and clusters were from Corning (New York, NY). [methyl-^{3}H]-Thymidine was from Amersham (Buckinghamshire, UK). Recombinant human tumor necrosis factor-a (TNF-a) was from Millipore (Billerica, MA). bFGF was from Sigma (St. Louis, MO). Antivimentin, and antidesmin were from Novocastra Laboratories (Newcastle, UK), antismooth muscle a-actin (a SMA) was from Sigma-Aldrich. Rabbit anti-b-actin was from Affinity Bioreagents (Rockford, IL). Horseradish peroxidase (HRP)-conjugated antibodies were from Pierce (Rockford, IL). zVAD-fmk was from Bachem (Bubendorf, Switzerland). Cyclosporin A (CsA) and propidium iodide were from Calbiochem (La Jolla, CA). Caspase-3, -8, and -9 Fluorometric kits were from R&D Systems (Minneapolis, MN). Invitrolon polyvinylidene difluoride (PVDF) membranes and NuPage gels were from Invitrogen (Carlsbad, CA). Rabbit poly (ADP-ribose) polymerase (PARP) antibody was from Cell Signaling (Danvers, MA). Goat procollagen Ia1 antibody and mAb to laminin γ were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antimicrotubule-associated protein light chain 3 (LC3) was from MBL (Naka-ku Nagaya, Japan). Goat antirabbit Alexa Fluor 488 was from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma-Aldrich.

Isolation and Culture of HIF

HIF were isolated from full thickness intestinal samples obtained from ileal, stenosing CD and ulcerative colitis (UC) patients undergoing surgical bowel resection, as well as from noninvolved, normal colon segments of patients undergoing resection due to colorectal cancer. Most CD patients requiring bowel surgery had received intense medical treatment and the decision to proceed to surgery was based, in most patients, on persistence of symptomatic bowel obstruction in the absence of significant inflammatory signs. All diagnoses were confirmed by clinical, radiologic, endoscopic, and histological criteria. HIF were isolated and cultured as previously described.[38] All experiments were per- formed with subconfluent cells at passage four or five. The project was approved by the local ethical committee and per- formed in accordance with the principles stated in the Decla- ration of Helsinki (Update October 1996).

Immunofluorescence Characterization of HIF

To characterize the phenotype of in vitro cultured HIF, cells were grown on coverslips and fixed in methanol at 20 C for 10 minutes, blocked in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) and 0.1% bovine serum albumin (BSA) for 1 hour, and incubated with primary antibodies, antivimentin (1:50), antismooth muscle a-actin (a-SMA) (1:200), or antidesmin (1:50) overnight. Then cells were incubated with fluorescent secondary antibody for 1 hour and mounted in Vectashield mounting medium with DAPI (Vector, Burlingame CA).

Treatment of HIF with TRF

TRF from palm oil (Tocomin 50%) was purchased from Carotech (Wendover, UK). Tocomin 50% is an oil suspension obtained from crude palm oil, which is extremely enriched in tocotrienols having the following vitamin E content: 11.1% α-tocotrienol, 2.1% β-tocotrienol, 20.8% γ-tocotrienol, 6.7% δ-tocotrienol, and 10.2% α-tocopherol.

Tocomin 50% was dissolved in ethanol to reach a 0.1% ethanol concentration in culture medium. Control cells were treated with 0.1% ethanol as vehicle. Twenty-four hours prior to commencing the experiments culture medium was replaced by Dulbecco's modified Eagle's medium (DMEM) medium with 0.3% FBS. Cyclosporin A, TNF-α, and cycloheximide were added at a 10 μM final concentration. zVAD-fmk was added at a 100 μM final concentration.

Proliferation Assay

Cells were plated in 12-well plates. After 24 hours, culture medium was replaced by serum-free medium containing the appropriate treatment and 0.25 μCi/mL [methyl-^{3}H]thymidine for an additional 24 or 48 hours. After this time, cells were washed twice with cold PBS and DNA was precipitated with ice-cold 5% trichloroacetic acid (TCA) for 15 minutes. Precipitates were dissolved in 0.5 M NaOH/0,1% SDS buffer and counted in a liquid scintillation analyzer.

DNA Fragmentation Assay

The extent of apoptosis in HIF was determined after 48 hours of treatment by the Cell Death Detection ELISA^{PLUS} assay from Roche (Mannheim, Germany) fol- lowing the manufacturer's instructions.

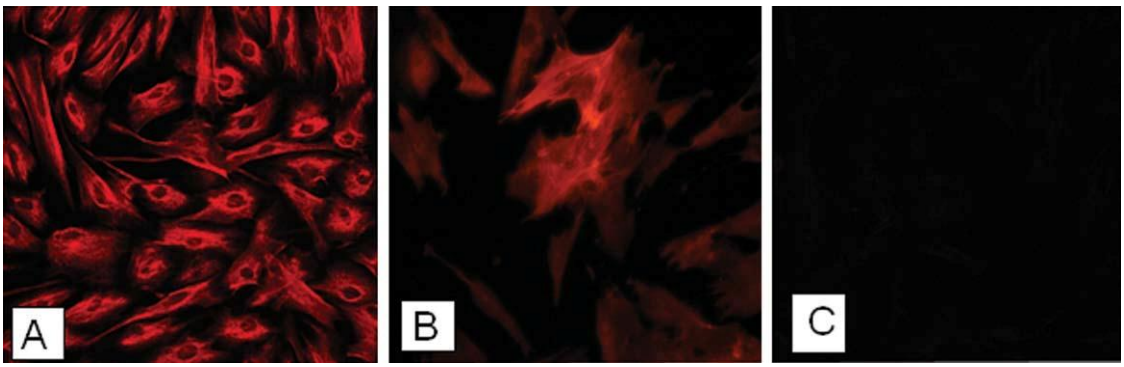

FIGURE 1. Immunofluorescence staining for (A) vimentin, (B) α-actin, and (C) desmin in cultured HIF cell lines.

Cell Cycle Analysis

Cells were treated for 48 hours and then harvested, washed in PBS, and fixed for 30 minutes in 70% ice-cold ethanol. After washing in cold PBS, cells were incubated in propidium iodide (PI) staining buffer (100 μg/mL PI, 250 μg/mL RNase A) for at least 30 minutes at 4 C and then analyzed by flow cytometry.

Caspase Activity Assay

Caspase activity was measured after 24-hour treatment with Caspase Fluorometric Assay kit (R&D Systems) following the manufacturer's instructions. Briefly, cells were harvested and lysed. After a 10-minute incubation at 4 C protein content was quantified with Bio-Rad Protein Assay (Munich, Germany) and 200 μg of protein per reaction were loaded. Reaction buffer was added with specific fluorogenic substrates for caspase-3 (DEVD-AFC), -8 (IETD-AFC), and -9 (LEDH-AFC). Caspase-dependent cleavage of 7-amino-4-trifluoromethyl coumarin (AFC) was measured in a Fluostar Optima microplate reader (BMG Labtechnologies, Offenburg, Germany) at Ex/Em 355/520 at 37 C for 2 hours.

Preparation of Whole Cell Lysates for Cytosolic Protein Detection

Cells were harvested with Trypsin-EDTA, washed with cold PBS, and lysed in ice-cold TLB buffer (20 mM Tris pH 7.4, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA) with Complete Protease Inhibitor Cocktail (Roche-Boehringer Mannheim, Germany) for 30 minutes. Cell lysates were centrifuged at 8500 rpm for 5 minutes and supernatants obtained for PARP, LC3, procollagen I α1, and laminin γ measurement.

Western Blot Analysis

Cell lysates were separated in a Nu-PAGE 3%-8% Tris-acetate gels with Tris-acetate SDS running buffer (for PARP, laminin γ and procollagen type I detection) or 12% Bis-Tris gels with 2-(N-morpholine)ethane sulfonic acid (MES-SDS) running buffer (for LC3 I/II) and transferred to PVDF membranes. The membranes were blocked and probed with antibodies against PARP (1/1000), laminin γ (1/100), procollagen type I (1/100), LC3I/II (1/2000), and β-actin (1/5000) with an overnight incubation at 4 °C and with HRP-conjugated antibodies for 1 hour at room temperature. Blots were developed using the Pierce Super-Signal WestFemto Maximum Sensitivity Substrate and bands were visualized using a Fujifilm Image Reader LAS-3000 phosphoimager (Fujifilm Photo Film, Tokyo, Japan). Quantification of protein expression was determined by densitometry of digitized images using Image Gauge V4.0 (Fujifilm Photo Film).

Detection of Autophagic Vacuoles

A total of 30,000 cells per well were plated onto 12-well plates and treated with TRF for 48 hours. After this time cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes, blocked for an additional 20 minutes, and incubated overnight with the primary anti-LC3 antibody (1/500 dilution in blocking buffer). Then cells were incubated with ALEXA 488 goat anti-rabbit (1/1000 dilution in blocking buffer) for 30 minutes and observed under a Nikon fluorescence microscope.

Statistical Analysis

Statistical analysis was performed using the Mann–Whitney test for nonparametric data and results are expressed as mean ± SEM. A P-value <0.05 was considered significant.

RESULTS

Immunofluorescence Characterization of HIF

Since various types of mesenchymal cells have been described in the intestine, we first aimed at describing the morphological characteristics of the HIF cell lines derived from surgical specimens (Fig. 1A–C). Using immunofluorescence to identify the presence of the cytoskeletal

markers vimentin, α-actin, and desmin, we could demonstrate that most cells (>95%) had a myofibroblast (vimentin, α-actin, desmin) phenotype, whereas almost no cells (<1%) had a smooth muscle phenotype (vimentin, α-actin, desmin) (Fig. 1A–C). No significant differences were observed in cell phenotype between normal, CD, and UC-derived HIF.

Spontaneous and bFGF-induced HIF Proliferation Is Reduced byT RF

To study the effect of TRF on HIF proliferation, a series of dose–response studies was carried out using [methyl-^{3}H] thymidine incorporation to the DNA. As shown in Figure 2A, treatment with 1 μM TRF had no effect on HIF proliferation. On the contrary, doses between 10 and 1000 μM TRF significantly reduced HIF proliferation with a plateau effect observed starting at 20 μM TRF. Results obtained with normal, CD, and UC HIF were identical and, therefore, combined results are displayed on Figure 2A, to underline the influence of TRF doses on HIF proliferation.

Next we investigated the ability of TRF to prevent bFGF-induced HIF proliferation. Interestingly, pretreatment with TRF 20 μM had a specific inhibitory effect on CD and UC, but not on control HIF proliferation (Fig. 2B).

TRF Induces HIF Apoptosis

Having demonstrated the effect of TRF on HIF proliferation, we next aimed at investigating whether TRF could also influence HIF apoptosis. First, we analyzed the ability of TRF to induce DNA degradation, using a DNA fragmentation assay. As shown in Figure 3A, TRF markedly increased DNA degradation in HIF, an effect of a similar magnitude as that induced by TNFα–Cycloheximide (CHX), a well-known proapoptotic stimuli. Similar results were obtained when HIFs were stained with PI and analyzed by flow cytometry (Fig. 3B,C). No significant differences on induction of cell apoptosis by TRF were observed between control, CD, and UC HIF (Fig. 3A,C).

TRF Induces Caspase Activation in HIF

Proapoptotic signals can be driven through the extrinsic and the intrinsic apoptotic pathways, which are governed by different patterns of caspase activation. To investigate the contribution of each of the main caspases to TRF-induced HIF apoptosis, we quantified activated caspase-3, -8, and -9 before and after exposure of HIF to TRF.

As shown in Figure 4, while unstimulated HIF displayed a very low degree of caspase activation, a marked activation of caspase-8 (Fig. 4A), caspase-9 (Fig. 4B), and caspase-3 (Fig. 4C) was observed in HIF upon TRF stimulation. Activation of caspase 3 can also be indirectly demonstrated by measuring PARP cleavage. PARP is involved in DNA repair in response to environmental stress[11] and is

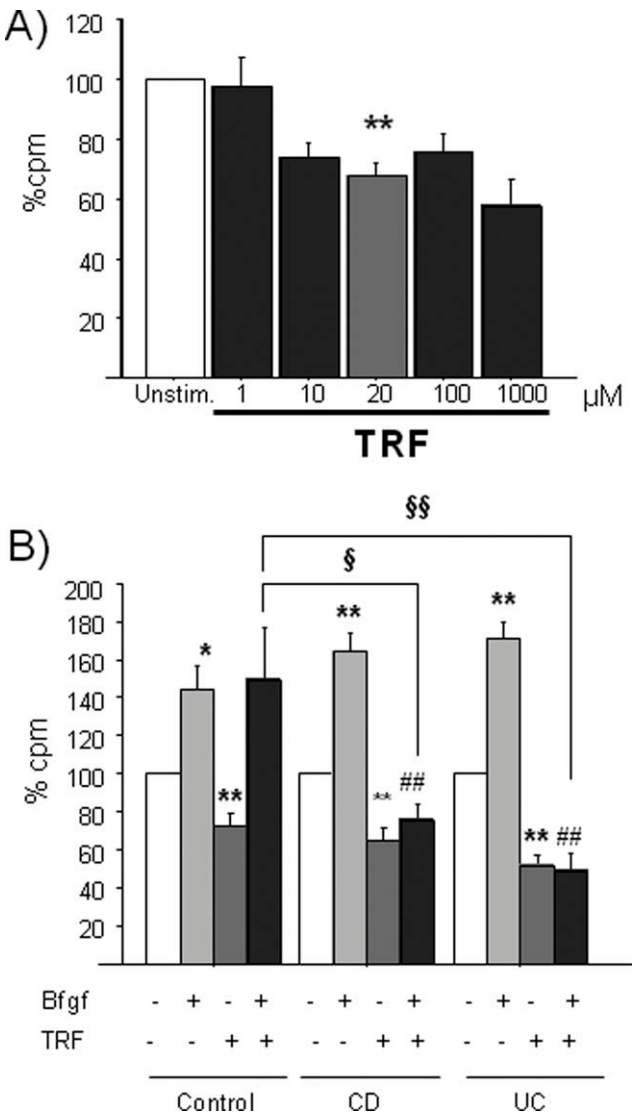

FIGURE 2. TRF inhibit proliferation of HIF. (A) Percentage of inhibition of proliferation with respect to unstimulated when cells were treated to different doses of TRF (1–1000 μM) as measured by [methyl-^{3}H] thymidine incorporation to the DNA following 24 hours treatment. Overall data are expressed as mean±SEM. (B) [methyl-^{3}H] Thymidine incorporation when cells were pretreated with TRF 20 μM for 1 hour following treatment with or without bFGF (10 ng) for an additional 24 hours. Data are presented as percentage of untreated cells and expressed as mean±SEM. *$P < 0.05$ versus unstimulated, **$P < 0.01$ versus unstimulated, ##$P < 0.01$ versus bFGF, §$P < 0.05$ versus control cells, §§$P < 0,01$ versus control cells; n=4.

one of the main cleavage targets of caspase-3 in vivo.[12] HIF-induced PARP degradation was apparent at 24 hours, peaked at 48 hours, and disappeared at 72 hours (Fig. 5A,B). As shown in Figure 5C, a similar degree of PARP degradation was observed between normal, CD, and UC HIF.

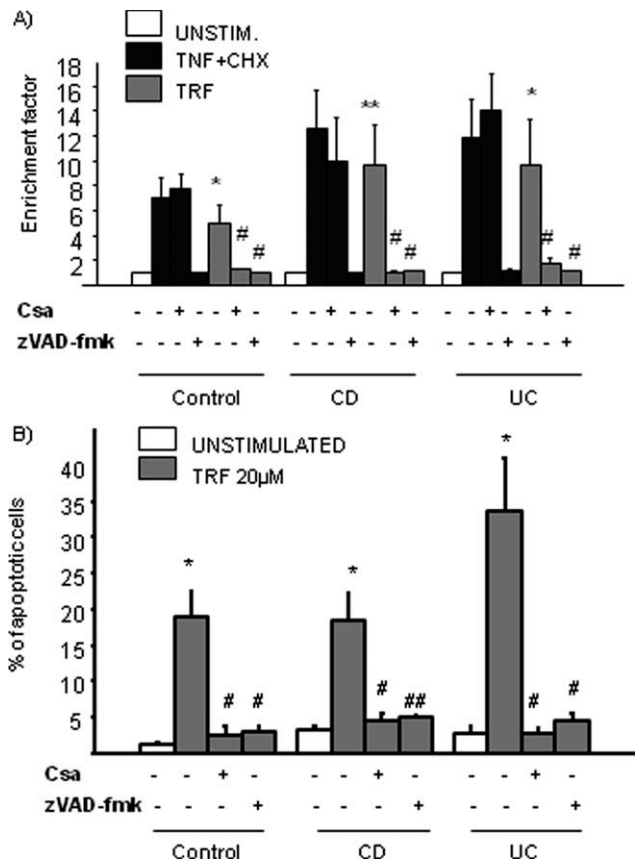

FIGURE 3. TRF induces apoptosis in cultured HIFs. (A) Cells were treated with TNF+CHX (10 μM) for 48 hours as a posi- tive control for apoptosis; alternatively, cells were treated with 20 μM with or without zVAD-fmk (100 μM) and CsA (10 μM) as potential apoptosis inhibitors. Data are pre- sented as mean±SEM and presented relative to unstimu- lated cells. *P < 0.05 versus unstimulated cells, **P < 0.01 versus unstimulated cells, #P < 0.05 versus TRF; n=5. (B) Graphic representation of apoptotic cells showing the effect of TRF, zVAD-fmk, and CsA on DNA degradation in different subpopulations of HIF. Data are presented as percentage of untreated cells and expressed as mean±SEM. *P < 0.05 versus unstimulated cells, #P < 0.05 versus TRF, ##P < 0.01 versus TRF; n=4.

Effect of zVAD-fmk and CsA on HIF Apoptosis and Caspase Activation

To demonstrate that TRF-induced apoptosis is a caspase-dependent process, we investigated the effect of the pancaspase inhibitor zVAD-fmk on each of the apoptosis-related outcomes described so far. zVAD-fmk was able to completely prevent both TRF and TNF+CHX-induced DNA degradation on normal, CD, and UC HIF as shown by the DNA degradation assay (Fig. 3A,C). Similarly, zVAD-fmk also completely abrogated TRF-induced caspase-8, -9, and -3 activation (Fig. 4), as well as TRF-induced PARP degradation in all types of HIF (Fig. 5B,C).

Next, we undertook a series of experiments pretreating HIF cultures with cyclosporine A (CsA) to block the mitochondrial permeability transition pore. Interestingly, CsA only prevented TRF-induced HIF apoptosis but not TNF+CHX-induced HIF apoptosis, which is developed through the extrinsic pathway (Fig. 3A,C). This selective

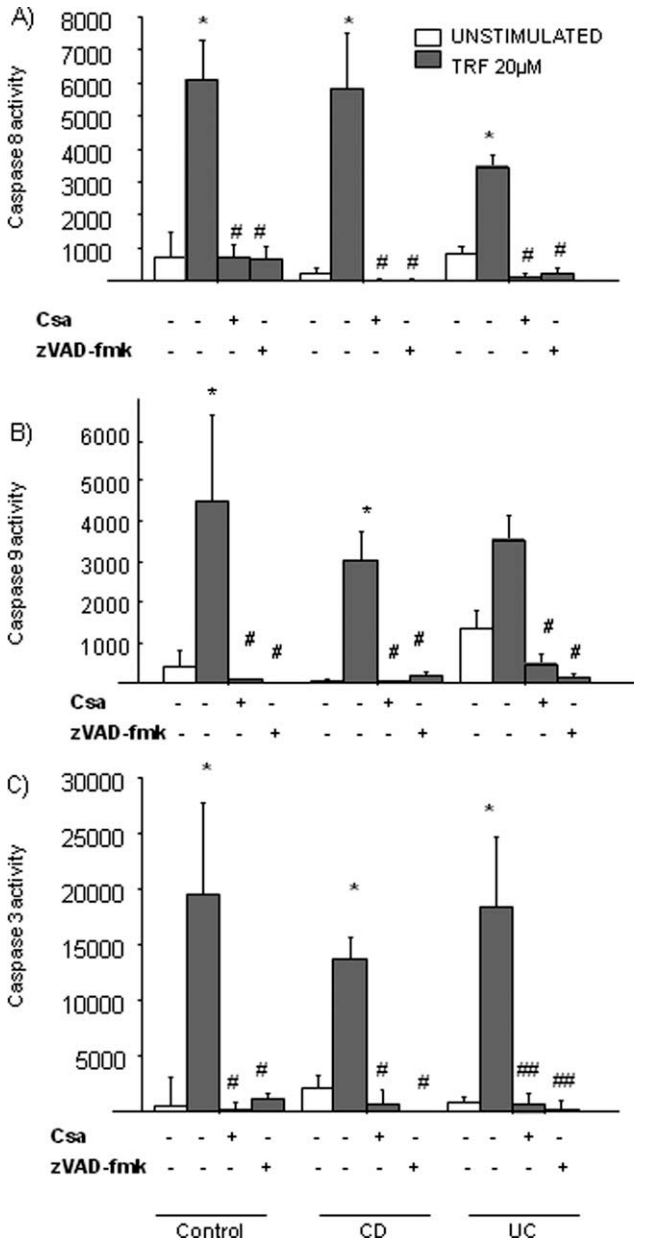

FIGURE 4. TRF activates extrinsic and intrinsic pathways of apoptosis. (A) Caspase-8, (B) caspase-9, and (C) caspase-3 measured, after 24 hours treatment, by fluorogenic assays in cell lysates using specific substrates as described in Materials and Methods. *P < 0.05 versus unstimulated cells, #P < 0.05 versus TRF, ##P < 0.01 versus TRF. Data are expressed as mean±SEM; n=3.

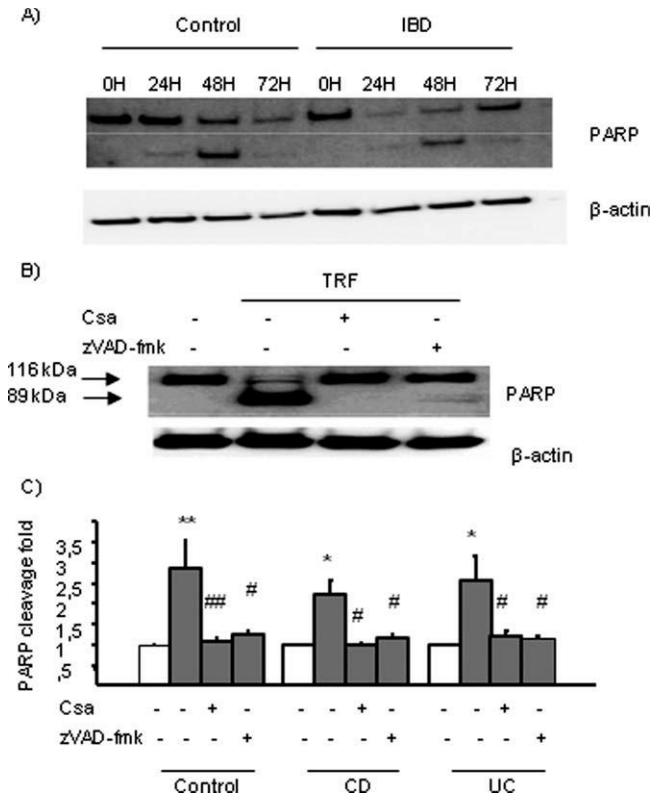

FIGURE 5. TRF triggers cleavage of PARP in a time-dependent manner. (A) Representative immunoblot analysis of PARP cleavage at the stated times. β-Actin is shown to demonstrate equal protein loading. (B) Representative immunoblot of PARP cleavage with the indicated treatments for 48 hours. (C) Graphic representation of PARP cleavage normal- ized with b-actin as determined by densitometry analysis of immunoblot for every HIF phenotype. Data are expressed as mean±SEM; n=3. *P < 0.05 versus unstimulated cells,**P < 0.01 versus unstimulated cells, #P < 0.05 versus TRF, ##P < 0.01 versus TRF.

effect suggests that the mitochondrial intrinsic apoptotic pathway plays a key role on TRF-induced HIF apoptosis.

TRF Induces HIF Autophagy

We investigated whether, in addition to HIF apoptosis, TRF was also able to induce HIF autophagy, an alternative type of programmed cell death. We used the microtubule-associated protein LC3 as a marker of HIF autophagy. Endogenous LC3 I, present in the cytoplasm, is processed to LC3 II and bound to the autophagosome membrane during the autophagy process. As shown by immunoblotting, TRF resulted in a pronounced accumulation of LC3 II clearly shown after 48 hours of HIF treatment (Fig. 6A,B). Analysis of control, CD, and UC HIF revealed no differences between these groups in response to TRF treatment (Fig. 6D). Induction of autophagy by TRF was also investigated using immunofluorescent detection of LC3, which demonstrated the presence of autophagic vacuoles on the cytoplasm of TRF-treated control, UC, and CD HIF, but not on untreated cells (Fig. 7). Of note, treatment of HIF with CsA completely prevented both LC3 maturation (Fig. 6C) and autophagic vacuole formation on HIF cytoplasm (Fig. 7), pointing to a critical involvement of the mitochondrial permeability on TRF-induced HIF autophagy. On the contrary, the pan-caspase inhibitor zVAD-fmk failed to prevent LC3 maturation and autophagic vacuole formation (Figs. 6, 7), thus proving that TRF is able to trigger caspase-dependent and -independent cell death pathways.

TRF Decreases HIF Matrix Protein Production

Increased production of ECM proteins is a key feature of the abnormal bowel fibrosis present in some CD patients. To investigate whether TRF treatment is also effective in preventing ECM deposition, we analyzed the production of procollagen type I and laminin γ before and after exposure of HIF to TRF, for different periods of time. As shown in Figure 8A,C, a marked reduction in both procollagen type I and laminin γ content was observed in HIF at 24 and 48 hours after TRF addition, being the reduction maximal at the later timepoint. TRF-induced reduction in ECM content was similar in control, CD, and UC HIF (Fig. 8B and 8D).

DISCUSSION

In this study we demonstrate that TRF can exert multiple effects on cultured HIF, including inhibition of cell proliferation, induction of programmed cell death through apoptosis and autophagy, and reduction of ECM production. Taken together, these effects point towards a potent antifibrogenic capacity of TRF that might be beneficial to treat bowel fibrosis or prevent its development in CD patients.

In the setting of persistent bowel inflammation, as in IBD, gut fibroblasts expand in number, contributing to the development of bowel fibrosis.[13] One of the key mediators that promote fibroblast proliferation is bFGF.[14,15] In the present study, TRF significantly reduced both spontaneous and bFGF-induced HIF proliferation. Interestingly, the inhibitory effect exerted by TRF was stronger in IBD than in control HIF. Moreover, TRF was able to completely abrogate bFGF-induced proliferation in UC and CD HIF, but not in control HIF. Considering that bFGF has been abundantly found in the colonic mucosa of IBD patients,[14,15] the ability to selectively inhibit bFGF-induced proliferation in inflamed HIF suggests a potential therapeutic benefit of TRF as an antifibrogenic drug.

It is well known that an exquisite equilibrium between cell proliferation and cell death is required to maintain physiological homeostasis. In that regard, the

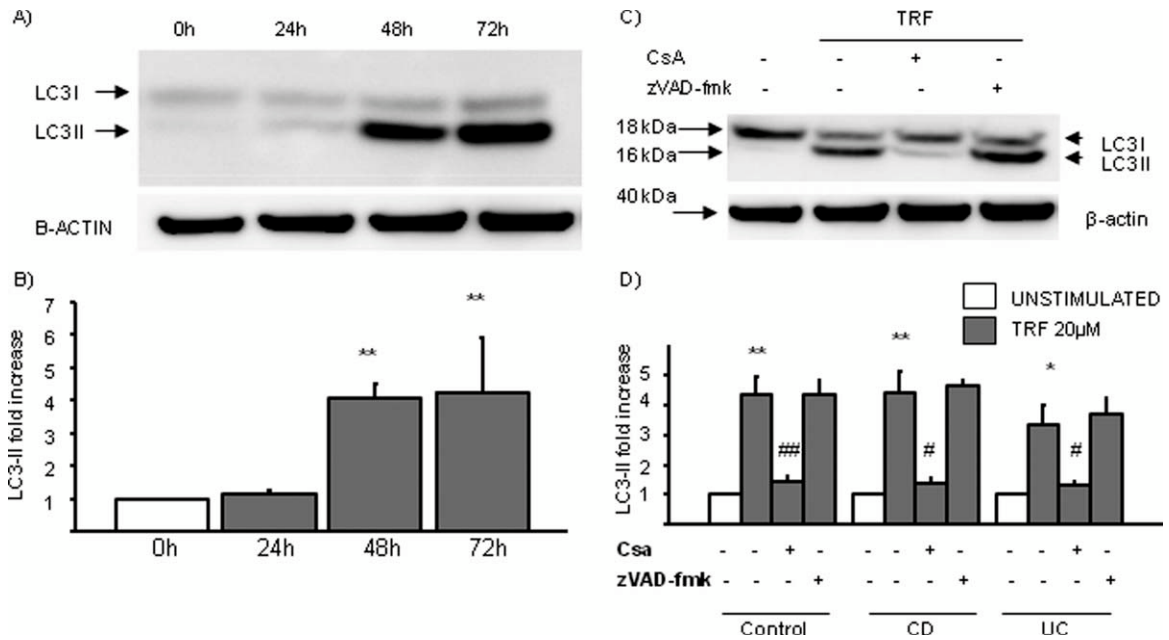

FIGURE 6. TRF induces HIF autophagy. (A) Representative immunoblot analysis of LC3-I (cytosolic form) and LC3-II (membrane-bound form) expression in cell lysates at the stated times. β-Actin is shown to demonstrate equal protein loading. (B) Graphic representation of LC3-II normalized with β-actin as determined by densitometry analysis of immunoblot shown in panel A. (C) Immunoblot showing the effect on LC3 maturation of zVAD-fmk and CsA on TRF-treated cells and (D) graphic quantification of LC3-II. Data are expressed as mean±SEM; n=4. *P < 0.05 versus unstimulated cells, **P < 0.01 versus unstimulated cells, #P < 0.05 versus TRF, ##P < 0.01 versus TRF.

excessive amount of fibroblasts observed in bowel fibrosis could result not only from increased cell proliferation but also from defective fibroblast apoptosis. In fact, the same concept has been proven for T lymphocytes in IBD. Excessive proliferation of T cells combined with a marked resistance of these cells to undergo apoptosis upon exposure to proapoptotic signals, such as Fas, nitric oxide, or IL-2 deprivation, leads to massive infiltration of the bowel wall by T lymphocytes in IBD patients.[16]

Interestingly, TRF was able to markedly enhance HIF apoptosis. Proapoptotic signals can be driven through two main apoptotic routes. The extrinsic pathway requires the

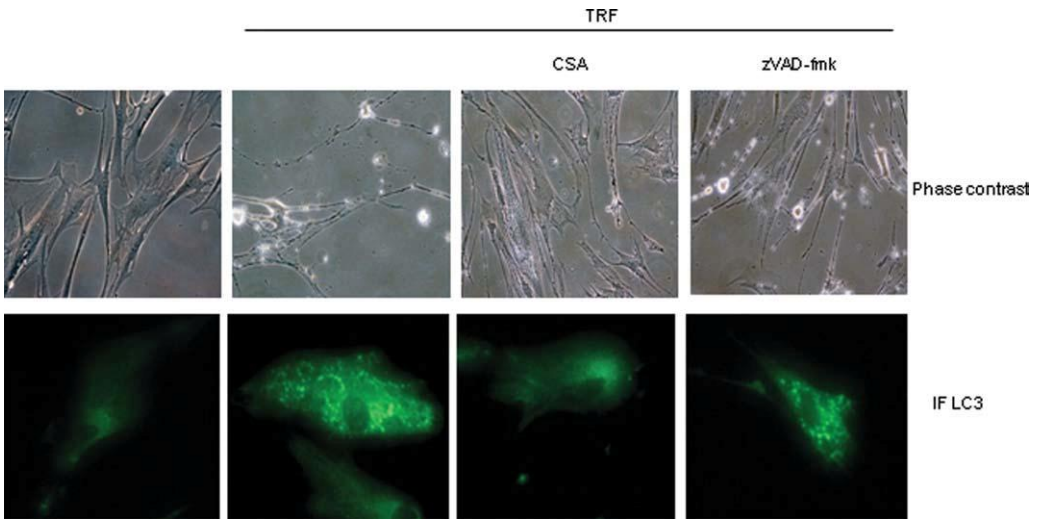

FIGURE 7. In vivo identification of LC3 II-labeled autophagolysosome vacuoles by fluorescence microscopy. Punctuate cytoplasmic vacuoles are observed in TRF and TRF+zVAD-fmk treated cells, whereas a diffuse background staining without cytoplasmic vacuoles is observed in untreated cells and TRF+CsA treated HIF (original magnification, 20).

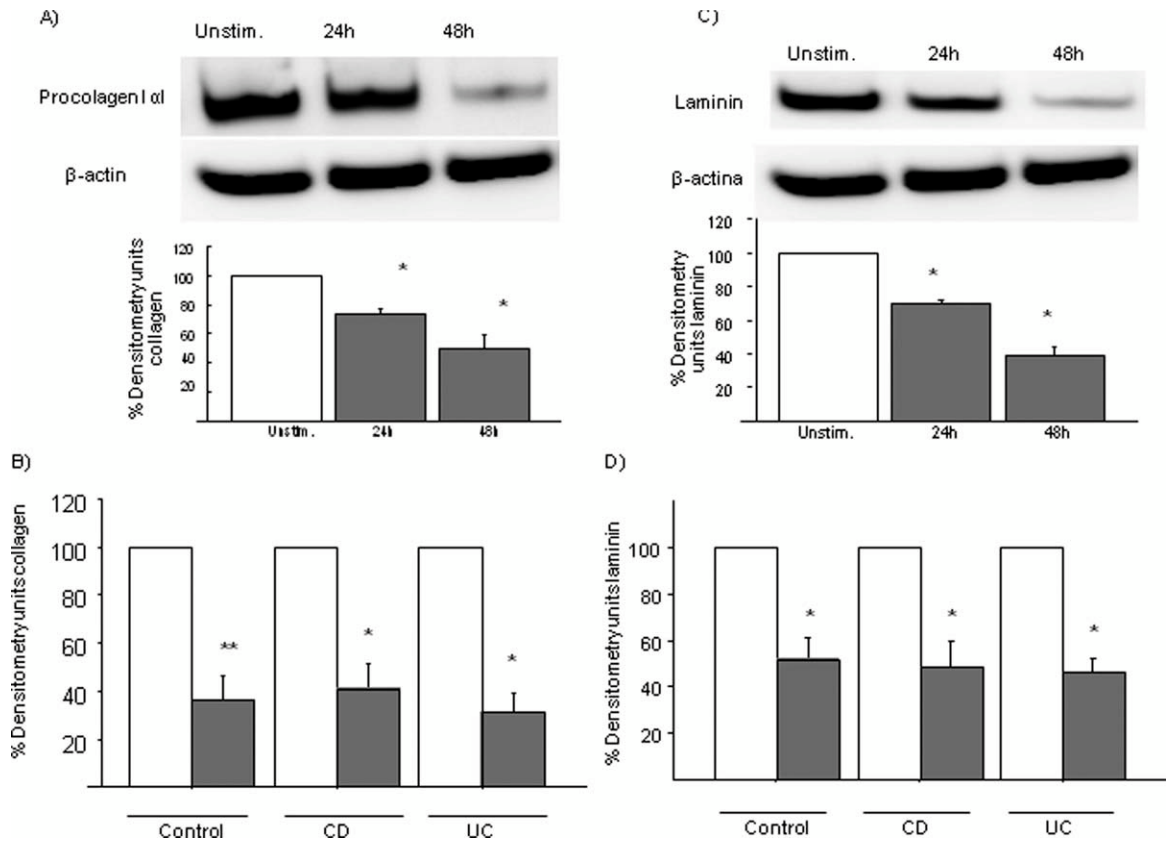

FIGURE 8. TRF decreases production of ECM proteins. (A) and (C) representative immunoblot and graphic representation showing a decrease in production of procollagen la1 and laminin γ at stated times. (B,D) Procollagen lα1 and laminin γ production after 48 hours of TRF treatment in each studied group of HIF. Data are expressed as mean±SEM. *P < 0.01 versus unstimulated cells, **P < 0.001 versus unstimulated cells.

binding of the proapoptotic agent to its membrane receptor, leading to caspase 8 activation, whereas the intrinsic pathway requires the participation of the mitochondria with release of mitochondrial cytochrome c and activation of caspase-9.[17] Both the extrinsic and intrinsic pathways converge in the activation of the effector caspase-3.[17,18] In addition to the existence of mono- and oligonucleosomes in the cytoplasm, the strong activation of caspases-3, -8, and -9, and the complete abrogation of HIF apoptosis by the pan-caspase inhibitor zVAD-fmk clearly demonstrated that TRF-induced apoptosis of HIF is indeed a caspase-dependent process.

In an attempt to dissect the specific contribution of each apoptotic pathway to TRF-induced HIF apoptosis, we undertook a series of experiments pretreating HIF with CsA, an inhibitor of the mitochondrial permeability transition pore that prevents the release of mitochondrial cytochrome c and the subsequent activation of caspase-9.[19] The TRF-induced marked activation of caspase-3, -8, and -9 was completely prevented by pretreatment of HIF with CsA, clearly demonstrating that TRF-induced apoptosis of HIF is predominantly mediated by the mitochondrial, intrinsic apoptotic pathway. In that regard, it is important to take into account that caspase-8, besides its orthodox activation by ligands of the TNF receptor family, can also be retroactivated by caspase-9 by means of a mitochondrial apoptotic loop.[20] This concept has been proven in different studies showing the inhibition of caspase-8 activation when cells were treated with CsA,[21,22] as in our study. Finally, activation of the effector caspase-3 was further demonstrated by measuring PARP cleavage. PARP, a 116-kDa nuclear poly (ADP-ribose) polymerase, is involved in DNA repair in response to environmental stress[11] and is one of the main cleavage targets of caspase-3 in vivo,[12] therefore resulting in a surrogate marker of cell apoptosis. As expected, addition of the pancaspase inhibitor zVAD-fmk to HIF completely prevented TRF-induced activation of caspases-3, -8, and -9, PARP cleavage, and cell apoptosis in HIF.

The capacity of inducing fibroblast apoptosis is likely to be essential for the efficacy of any antifibrogenic treatment. This notion is supported by the fact that most efficacious drugs to treat bowel inflammation, such as steroids, azathioprine, methotrexate, infliximab, and adalimumab,

have been shown to be potent inductors of immune cell apoptosis.[23–25] In keeping with this concept, it has been recently shown that apoptosis of activated hepatic stellate cells, the functional equivalent to intestinal fibroblasts, is required to achieve regression of liver fibrosis.[26,27] These results constitute the rationale supporting the provocative view that tissue fibrosis might be a reversible state and has motivated intense research efforts aimed at identifying new molecules capable of promoting fibroblast apoptosis.

One of the most relevant findings of our study was the demonstration that TRF, in addition to apoptosis, can also induce autophagy in HIF. Exposure of HIF to TRF resulted in a markedly increased expression of LC3 II, a membrane-bound protein used to monitor autophagy that is involved in the generation of autophagosomes[28] and also in the number of autophagic vacuoles in HIF cytoplasm, as demonstrated by fluorescent microscopy. Interestingly, TRF-induced HIF autophagy was completely prevented by pretreatment of HIF with CsA, but was unaffected when HIFs were pretreated with the pan-caspase inhibitor zVAD-fmk, demonstrating that TRF-induced HIF autophagy is a caspase-independent process. Autophagy is a constitutive process required for proper cellular homeostasis and organelle turnover.[29] However, the true relevance of autophagy in CD pathophysiology has been very recently unveiled by genome-wide association scan studies (GWAS). Several GWAS studies have found a strong association between genetic variants in two autophagy genes, ATG16L1 and IRGM, and an increased risk to develop CD.[30–32]

Along with fibroblast accumulation, an excessive deposition of ECM is also a hallmark of abnormal tissue fibrosis. TRF markedly decreased the amount of procollagen type I and laminin c-1 in HIF, two main components of ECM that have been found abundantly expressed in bowel strictures of CD patients.[1,33,34] Interpretation of the global impact of TRF on ECM production by HIF is complex. Induction of programmed cell death through apoptosis and autophagy could, by itself, reduce the amount of ECM produced by HIF. However, it must be underlined that during these processes HIFs were still able to upregulate the expression of certain proteins, such as caspases or LC3II, whereas the ECM components procollagen type I and laminin c-1 were downregulated. Therefore, it is likely that both the reduction in HIF number combined with specific downregulation of ECM components would contribute to the marked decrease in ECM deposition by HIF, enhancing the antifibrogenic potential of TRF.

In the present study we found relatively few differences among CD, UC, and control HIF in response to TRF. Although we demonstrated indeed a selective capacity of TRF to prevent bFGF-induced proliferation only in CD and UC, but not in control HIF, no differences were observed among the three cell types in other outcomes. The notion that TRF predominantly exerts its antiproliferative and proapoptotic effects on activated cells is at present widely accepted and has been previously demonstrated by our group[10] and others.[9,35,36] Underlying the influence of the degree of cell activation on TRF effects, it has been recently reported that TRF inhibits proliferation and induces apoptosis in the human colon carcinoma cell line HT-29.[36] We hypothesized that the experimental setting used
has contributed to the lack of differences among the three HIF groups. A prolonged period of time, ranging between 1 and 2 months, is required to obtain primary cultures of HIF from intestinal samples and it has been demonstrated that during this process fibroblasts experience a significant degree of activation.[37]

In our study we could not study the effects of TRF on freshly isolated intestinal epithelial cells. It is well known that, upon detachment from the basal membrane, intestinal epithelial cells undergo apoptosis very rapidly, which made impossible the study of proliferation, apoptosis, or autophagy in these cells. However, in a previous study our group demonstrated that TRF does not induce any type of programmed cell death in quiescent, acinar pancreatic cells, a type of epithelial cells isolated from the rat pancreas, whereas TRF was able to induce apoptosis in pancreatic fibroblasts isolated from the same animals.[10] In conclusion, our study demonstrates that TRF has multiple antifibrogenic effects on HIF, derived from its capacity to reduce cell proliferation, induce programmed cell death, and inhibit ECM production by HIF. Considering also its excellent safety profile, TRF represents a promising therapeutic strategy to treat bowel fibrosis or to prevent its development in CD patients.

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2) Segon treball

Palm oil tocotrienol rich fraction reduces extracellular matrix production by inhibiting transforming growth factor-β1 in human intestinal fibroblasts

El TGF-β té un paper molt important en el desenvolupament dels processos que cursen amb fibrosi, ja que promou la síntesi de MEC alhora que n'inhibeix la seva degradació. L'objectiu d'aques estudi és esbrinar els efectes de la FRT en la síntesi de MEC i en la producció de proteïnes que en regulen la degradació com MMP-3 i TIMP-1 per part dels FIH, i si els efectes de la FRT estan mediats mitjançant la inhibició de la senyalització per TGF-β.

La FRT disminueix significativament la producció de pro-col·làgen 1 i 3 en FIH.

La FRT incrementa la producció de MMP-3 encara que no modifica la producció de TIMP-1.

El pre-tractament de FIH amb FRT disminueix la fosforilació de Smad3 i minimitza l'increment en la producció de col·làgen 1 i 3 causat per TGF-β1.

PALM OIL TOCOTRIENOL RICH FRACTION REDUCES EXTRACELLULAR MATRIX PRODUCTION BY INHIBITING TRANSFORMING GROWTH FACTOR-B1 IN HUMAN INTESTINAL FIBROBLASTS

Short title: Tocotrienols reduce extracellular matrix production

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Abstract

Background & Aims: Extracellular matrix deposition is key event for the development of bowel stenosis in Crohn's disease patients. Transforming growth factor-β plays a key role in this process. We aimed at characterizing the effects of tocotrienol rich fraction on ECM proteins production and molecules that regulate the synthesis and degradation of extracellular matrix, matrix metalloproteinase-3 and tissue inhibitor of metalloproteinases-1, in human intestinal fibroblasts, and at elucidating wether the effects of tocotrienol rich fraction (TRF) are mediated through inhibition of TGF-β1. **Methods:** HIF were isolated from colonic or ileal tissue from Crohn's disease patients and control subjects, and were treated with TRF from palm oil either alone or in combination with TGF-β1. Procollagen 1, procollagen 3, TIMP-1 and MMP-3 production, and Smad3 phosphorylation were analized by Western-blotting. **Results:** TRF significantly diminished pro-collagen 1 and 3 synthesis in HIF. Treatment of HIF with TRF increased MMP-3 production but did not modify TIMP-1. TGF-β1 induced Smad3 phosphorylation and enhanced procollagen 1 and 3 and TIMP-1 production. Pre-treatment of HIF with TRF prevented Smad3 phosphorylation and minimized the increase in collagen 1 and 3 production caused by TGF-β1. **Conclusions:** TRF has antifibrogenic effects on HIF, decreasing ECM production and increasing its degradation. This effect is mediated, at least in part, by inhibition of TGF-β1.

Key Words: inflammatory bowel disease, fibroblasts, tocotrienol rich fraction.

Introduction

Crohn's disease (CD) is a very heterogeneous condition and approximately half of CD patients will develop a stenosing phenotype, characterized by progressive narrowing of the intestinal lumen[1]. In these patients abnormal bowel fibrogenesis is due to chronic transmural inflammation and impaired wound healing, which result in massive fibroblast proliferation and an excessive deposition of extracellular matrix (ECM) in the bowel wall. Ultimately, abnormal contraction of the ECM will also contribute to the tissue distortion and intestinal obstruction.

No medical treatment for bowel fibrosis has become available to date, in spite of the remarkable success of the new, anti-inflammatory therapies recently developed for inflammatory bowel disease (IBD)[2]. Due to the lack of medical therapies for bowel fibrosis most CD patients with a stenosing phenotype will require surgical resection of the involved bowel segment, either once or often more times during their live.

Relatively minor progress has been made in our knowledge of the molecular mechanisms that lead to bowel fibrosis, as compared to liver, lung, kidney or skin fibrosis[3]. Several molecules have been shown to be involved in the abnormal bowel fibrogenesis that takes place in stenosing CD patients. Among them, transforming growth factor-β (TGF-β) seems to play a key role in this process[4]. TFG-β is highly expressed in areas of intestinal stricture and is overproduced by fibroblasts isolated from enteric strictures[5]. In particular, the TGF-β1 isoform has been specifically implicated in fibrosis through its ability to promote ECM synthesis and fibroblast contraction[6]. Both TGF-β and its receptors are overexpressed in the intestine of patients with CD[7], and their binding induces the activation of the Smad transcriptional proteins. TGF-β receptor I kinase directly phosphorylates Smad2 and Smad3 that then bind to the

common mediator Smad4 and translocate to the nucleus to regulate gene transcription. The inhibitory Smad proteins, Smad6 and Smad7, compete for the TGF-β receptor I kinase and inhibit Smad3 phosphorylation. Moreover, TGF-β1 may facilitate the fibrogenic process by stimulating tissue inhibitor of metalloproteinases-1 (TIMP-1) production or inhibiting matrix metalloproteinases (MMP) expression[8]. Excessive synthesis and deposition of ECM components by intestinal myofibroblasts, as well as inhibition of ECM degradation, due to an imbalance between MMPs and their inhibitors, TIMPs, are thought to be involved in bowel fibrogenesis[9].

A variety of natural dietary constituents, including vitamin E, have recently attracted the researcher's attention for their potential health benefits and harmless consumption profile. In nature, eight substances have been found to have vitamin E activity: α-, β-, γ- and δ-tocopherol; and α-, β-, γ- and δ-tocotrienol. Dietary tocotrienols are well absorbed and easily distributed throughout the body tissues. These vitamin E derivatives could provide health benefits due to their antiproliferative, neuroprotective, and cholesterol-lowering properties[10-12]. The suppressive effects of tocotrienols on tumour growth are attributed to their ability to induce both cell cycle arrest and apoptosis in transformed cells[13]. On the contrary, α-tocopherol is not effective in inducing apoptosis in cancer cells[14]. Two previous studies from our group demonstrated that tocotrienols can induce programmed cell death through apoptosis and autophagy in rat pancreatic stellate cells[15] and human intestinal fibroblasts (HIF), in vitro[16].

The purpose of the present study was to characterize the effects of tocotrienols on ECM production by HIF and to investigate whether such effects are mediated by inhibition of the TGF-β1 profibrogenic effects on these cells.

Materials and Methods

Reagents and antibodies

Cell culture flasks and clusters were from Corning (New York, NY). Tocotrienol Rich Fraction from palm oil (Tocomin 50%) was kindly provided by Carotech Ltd (Wendover, UK). Rabbit anti-β-actin and recombinant human TGF-β1 were from Sigma-Aldrich (St. Louis, MO). Monoclonal TGF-β1 antibody, rabbit pSmad3 antibody, mouse monoclonal Smad7 antibody and goat matrix metalloproteinase-3 (MMP-3) antibody were from R&D Systems (Minneapolis, MN). Monoclonal TIMP-1 antibody was from Calbiochem (Nottingham, UK). Invitrolon polyvinylidene difluoride (PVDF) membranes and NuPage gels were from Invitrogen (Carlsbad, CA). Mouse monoclonal Smad3 antibody, goat procollagen 1α1 antibody, rabbit procollagen 3α1 and horseradish peroxidase (HRP)-conjugated antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Isolation and culture of HIF

HIF were isolated from intestinal segments of patients with stenosing CD undergoing surgical bowel resection, as well as from non-involved, normal colon segments of patients undergoing resection due to colorectal cancer. All diagnoses were confirmed by clinical, radiologic, endoscopic, and histological criteria. HIF were isolated and cultured as previously described[17]. All experiments were performed with subconfluent cells at passage five. The project was approved by the local ethical committee and performed in accordance with the principles stated in the Declaration of Helsinki (Update October 1996).

Treatment of HIF with TRF

TRF is an oil suspension obtained from crude palm oil, which is extremely enriched in tocotrienols having the following vitamin E content: 11.1% α-tocotrienol, 2.1% β-tocotrienol, 20.8% γ-tocotrienol, 6.7% δ-tocotrienol, and 10.2% α-tocopherol. TRF was dissolved in ethanol to reach a 0.1% ethanol concentration and a 20 μM concentration of TRF in culture medium. TRF dose was chosen according to our previous publications[12, 13]. Control cells were treated with 0.1% ethanol as vehicle. Experiments were performed in DMEM medium with 0.3% FBS. Recombinant TGF-β1 was added at a 10 ng/mL final concentration.

Preparation of Whole cell lysates for cytosolic protein detection

Cells were harvested with Trypsin-EDTA, washed with cold PBS, and lysed in ice-cold TLB buffer (20 mM Tris pH 7.4, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2mM EDTA) with Complete Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail (Roche-Diagnostics, Mannheim) for 30 min. Cell lysates were centrifuged at 8500 rpm for 5 min and supernatants obtained for pSmad3, Smad3, Smad7, TIMP-1, MMP-3, procollagen 1α1 and procollagen 3α1 measurement.

Western Blot Analysis

Cell lysates were separated in a Nu-PAGE 3%-8% tris-acetate gels with tris-acetate SDS running buffer (for procollagen type I and procollagen type III detection), 4-12% Bis-Tris gels with MOPS-SDS running buffer (for pSMAD3, SMAD3, SMAD7 and MMP-3 detection) or 12% Bis-Tris gels with MES-SDS running buffer (for TIMP-1 detection) and transferred to PVDF membranes. The membranes were blocked and

probed with antibodies against pSmad3 (1/500), Smad3 (1/200), Smad7 (1/500), MMP-3 (1/2500), TIMP-1 (1/250), procollagen type 1 and type 3 (1/100) and β-actin (1/5000) with an overnight incubation at 4ºC and with HRP-conjugated antibodies for 1 hour at room temperature. Blots were developed by using the Pierce SuperSignal WestFemto Maximum Sensitivity Substrate (Pierce, Rockford, IL) and bands were visualized by using a Fujifilm Image Reader LAS-3000 phosphoimager (Fujifilm Photo Film Co., Tokyo, Japan). Quantification of protein expression was determined by densitometry of digitalized images by using Image Gauge V4.0 (Fujifilm Photo Film Co., Tokyo, Japan).

Statistical Analysis

Statistical analysis was performed using the Mann-Whitney test for non-parametric data, and results are expressed as mean ± SEM. A P value <0.05 was considered significant.

Results

TGF-β1-mediated HIF intracellular signalling

In most cell types TGF-β1 intracellular signalling is driven by the phosphorylation of Smad3, which results in the activation of this pro-fibrogenic mediator, and its negative regulator, Smad7. When HIF were exposed to TGF-β1, strong Smad3 phosphorylation was induced, with a peak of activation observed 6 hours after TGF-β1 treatment. Smad3 phosphorylation remained up-regulated at 24 and 48 hours, compared to baseline. Paralleling Smad3 phosphorylation, total Smad3 decreased upon TGF-β1 treatment. The inhibitor of Smad3 phosphorylation, Smad7, was only slightly up-regulated after 6 hours of TGF-β1 treatment, but its levels were rapidly normalized at later time points (**Fig. 1A and 1B**).

Effect of TRF on TGF-β1-mediated HIF intracellular signalling

Having shown that TGF-β1 induces a marked activation of Smad3, which will ultimately lead to its pro-fibrogenic effects, we next aimed at studying the ability of TRF to interfere with that process. Treatment with TRF alone had no effect on Smad3 activation, but increased the levels of the inhibitory regulator Smad7 in HIF, compared to unstimulated cells. Pre-treatment of HIF with TRF, prior to TGF-β1 stimulation, was able to markedly reduce TGF-β1-induced Smad3 phosphorylation, without modifying Smad7 expression (**Fig. 1C and 1D**). When TRF and TGF- β1 were simultaneously administered no effect of TRF on TGF-β1-induced Smad3 phosphorylation was observed (data not shown).

TGF-β1-induced TIMP-1 and MMP-3 production by HIF

TGF-β1 is able to influence the expression of both metalloproteinases and their inhibitors in various cell types, usually favouring the deposition of extracellular matrix. TGF-β1 significantly increased TIMP-1 production in HIF, without altering the levels of MMP-3 over time, therefore shifting the MMP-3 / TIMP-1 ratio towards a lower metalloproteinase production (**Fig 2**).

Effect of TRF on TGF-β1-induced TIMP-1 and MMP-3 production by HIF

We next aimed at ascertaining whether the changes induced by TRF on the Smad3 / Smad7 HIF intracellular signalling, shown above, have an influence on MMP-3 and TIMP-1 production by HIF. Interestingly, treatment of HIF with TRF alone significantly increased MMP-3 production whereas TIMP-1 levels remained unchanged (**Fig 3A**), thus increasing the ratio MMP-3 / TIMP-1 and favouring the extracellular matrix degradation. Moreover, when used in combination with TGF-β1, TRF was also able to markedly increase MMP-3 expression without changing TIMP-1 expression, therefore counterbalancing the pro-fibrogenic effects of TGF-β1 on the MMP-3 / TIMP-1 ratio in HIF (**Fig 3**).

TRF decreases TGF-β1-induced ECM protein production

TGF-β1 is a key regulator of ECM production, favouring its deposition. Treatment with TGF-β1 promoted indeed procollagen type 1 and procollagen type 3 synthesis in a time dependent manner in HIF (data not shown). Notably, treatment of HIF with TRF was able to completely abrogate TGF-β1-induced procollagen type 1 up-regulation (**Fig 4**). TRF also tend to have an inhibitory effect on TGF-β1-induced procollagen type 3 production, but differences did not reach statistical significance (**Fig. 4**). Finally, HIF derived from fibrotic areas of CD patients showed higher procollagen type 1 production

than control HIF upon TGF-β1 stimulation (**Fig. 5A**). No differences were observed in the case of procollagen type 3 production (data not shown). Interestingly, TRF was also able to completely abrogate TGF-β1-induced procollagen type 1 up-regulation in CD HIF, resulting in procollagen type 1 levels similar to those of untreated, control HIF (**Fig. 5B**).

Discussion

In this study, we demonstrate that TRF have profound effects on HIF, attenuating TGF-β1-mediated Smad3 phosphorylation, up-regulating MMP-3 production and inhibiting TGF-β1-mediated collagen type 1 and 3 synthesis by these cells. Taken together, these effects may shift the balance between ECM production and degradation by HIF, counteracting the pro-fibrogenic effects of TGF-β1 and favouring ECM degradation.

TGF-β is a multifunctional cytokine regulating a variety of biological responses including cell growth and differentiation, apoptosis, cell migration, immune cell function and extracellular matrix production[18]. Many fibrotic pathologies, including CD, are associated with increased levels of TGF-β which result in fibroblast recruitment in injured tissues and subsequent fibroblast stimulation and ECM production. We have shown for the first time that TRF has the ability to influence TGF-β1-mediated intracellular signalling in HIF. TRF markedly decreased the activation of Smad3, a key mediator of TGF-β1 pro-fibrogenic effects. On the contrary, TRF did not affect Smad7 production. Smad7 is an inhibitory Smad that binds to the Type I receptor preventing recruitment and phosphorylation of Smad3, and results in a net inhibition of TGF-β1-mediated signalling. The relevance of these effects is underlined by the observation that deletion of Smad3 completely abrogates TGF-β1-mediated production of different types of collagen by fibroblasts[19, 20]. Our results are in keeping with previous studies, demonstrating that certain vitamin E derivatives, such as α-tocopherol, may influence cell signalling by inhibiting protein kinase C in vascular smooth vascular cells[21, 22]. Similarly, α-tocopherol has also been shown to regulate Akt/PKB activation[23]. The ability of TRF, α-tocopherol and other vitamin E derivatives to influence cell signalling is related to their hydrophobic properties that determine their location in the cell

membranes where they form complexes with lipid rafts, cholesterol and sphingolipid enriched microdomains, serving as a platform for signalling complexes[24].

TRF was also able to enhance MMP-3 without altering TIMP-1 synthesis by HIF, therefore shifting the balance between these two key regulators of ECM content towards ECM degradation. Metalloproteinases and their inhibitors are a family of proteins with a key role governing the synthesis and degradation of ECM in various tissues. In a murine model of chronic intestinal inflammation, fibrosis was associated with an increase in TIMP-1 expression which resulted in inhibition of MMP-mediated ECM degradation[25]. Increased TIMP-1 has also been shown in collagenous colitis and colonic diverticular disease[26, 27], two entities characterized by marked intestinal fibrosis. Furthermore, in strictured intestinal segments of CD patients TIMP-1 expression has been found up-regulated and MMP-3 and MMP-12 down-regulated, compared to non strictured areas[28].

In sharp contrast to our results, another vitamin E derivative, α-tocopherol, diminished collagenase gene transcription without altering the level of its natural inhibitor, TIMP-1, in human skin fibroblasts[29], and down-regulated MMP-19 in myeloid cells[30].

Along with fibroblast accumulation, an excessive deposition of ECM is also a hallmark of abnormal tissue fibrosis. TRF was also able to profoundly modify TGF-β1-mediated ECM production of procollagen type 1 in HIF, two main components of ECM that have been found abundantly expressed in bowel strictures of CD patients[3, 31]. Accumulation of ECM in fibrotic diseases usually results from elevated mRNA levels of fibrillar collagens due to increased transcriptional activation. The fibrotic mucosa of patients with IBD shows increased levels of mRNA and protein of collagen types I, III, IV and V[32]. TGF-β plays a central role in modulating ECM gene expression through Smad3 signalling. A number of collagen gene promoters are induced by TGF-β1 and are

dependent upon Smad3 in dermal fibroblasts. These include COL1A1, COL1A2, COL3A1, COL5A2, COL6A1 and COL6A3[33].

One of the most relevant findings of our study is the demonstration that HIF isolated from CD strictures produce more procollagen type 1 than control HIF upon TGF-β1 stimulation. Interestingly, TRF was able to completely abrogate TGF-β1-mediated procollagen type 1 upregulation in CD and control HIF. As recently shown by our group, TRF is also able to markedly reduce proliferation and enhance programmed cell death through apoptosis and autophagy in HIF, which ultimately will also contribute to the anti-fibrogenic potential of TRF[15, 16]. The impact of TRF on the number of HIF could, by itself, reduce the amount of ECM produced by these cells. However, it must be underlined that upon treatment with TRF HIF are still able to up-regulate the expression of certain proteins, such as MMP-3, whereas the ECM components procollagen type 1 and procollagen type 3 are down-regulated. We therefore conclude that both, a reduction in HIF number resulting from a decreased proliferation and an increased programmed cell death and the specific down-regulation of ECM components, contribute to the observed TRF-induced reduction in ECM deposition by HIF.

Furthermore, dietary vitamin E supplementation in human subjects and animal models has an antioxidant and antiinflammatory effect. Supplementation decreases C-reactive protein (CRP) and release of proinflammatory cytokines[35].

The present work completes our previous description of the “in vitro” effects of TRF on HIF. Thus, while in our previously published paper we basically described a quantitative anti-fibrogenic effect or net reduction in the number of HIF cells resulting from combined reduction of HIF proliferation with enhancement of HIF programmed cell death through both apoptosis and autophagy, in the present work we characterize a

more qualitative antifibrogenic effect, inhibiting TGF-β1-mediated signalling and profibrogenic effects.

In conclusion, our study demonstrates that TRF has multiple anti-fibrogenic effects in HIF, derived from its capacity of inducing ECM degradation through the MMP / TIMP system and counteracting TGF-β1 pro-fibrogenic actions. Considering also its excellent safety profile, TRF represents a promising therapeutic strategy to treat bowel fibrosis or to prevent its development in CD patients.

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Figure legends

Figure 1. (A) Time-course of transforming growth factor-β1 (TGF-β1) mediated intracellular signalling on human intestinal fibroblasts (HIF). HIF were stimulated with 10 ng/mL over time. Phosphorylation of Smad3 and total amounts of Smad3, Smad 7 and β-actin were determined by Western blot analysis. Blots are representative of three independent experiments. (C) Effect of tocotrienol rich fraction (TRF) on transforming growth factor-β1 (TGF-β1) mediated intracellular signalling in human intestinal fibroblasts (HIF). HIF were pretreated with 20 μM TRF for 24 hours and with TGF-β1 for 6 hours. Unphosphorylated and phosphorylated Smad3 and Smad7 were assessed by Western blot. All blots are representative of three independent experiments. (B) and (D) Quantification of pSmad3, Smad3 and Smad7, normalized by β-actin as determined by densitometric analysis of each immunoblot. Data are expressed as mean ± SEM; $n \geq 3$. *$P < 0.05$ vs. unstimulated cells, #$P < 0.05$ vs. TGF-β1.

Figure 2. Effect of transforming growth factor-β1 (TGF-β1) on tissue inhibitor of metalloproteinases-1 (TIMP-1) and matrix metalloproteinase-3 (MMP-3) production by human intestinal fibroblasts (HIF) over time. (A) Representative immunoblot of TIMP-1 and MMP-3 production by HIF upon TGF-β1 stimulation. (B) Quantification of TIMP-1 production by HIF, normalized by β-actin as determined by densitometric analysis of each immunoblot. Data are expressed as mean ± SEM; $n \geq 3$. *$P < 0.05$ vs. unstimulated cells.

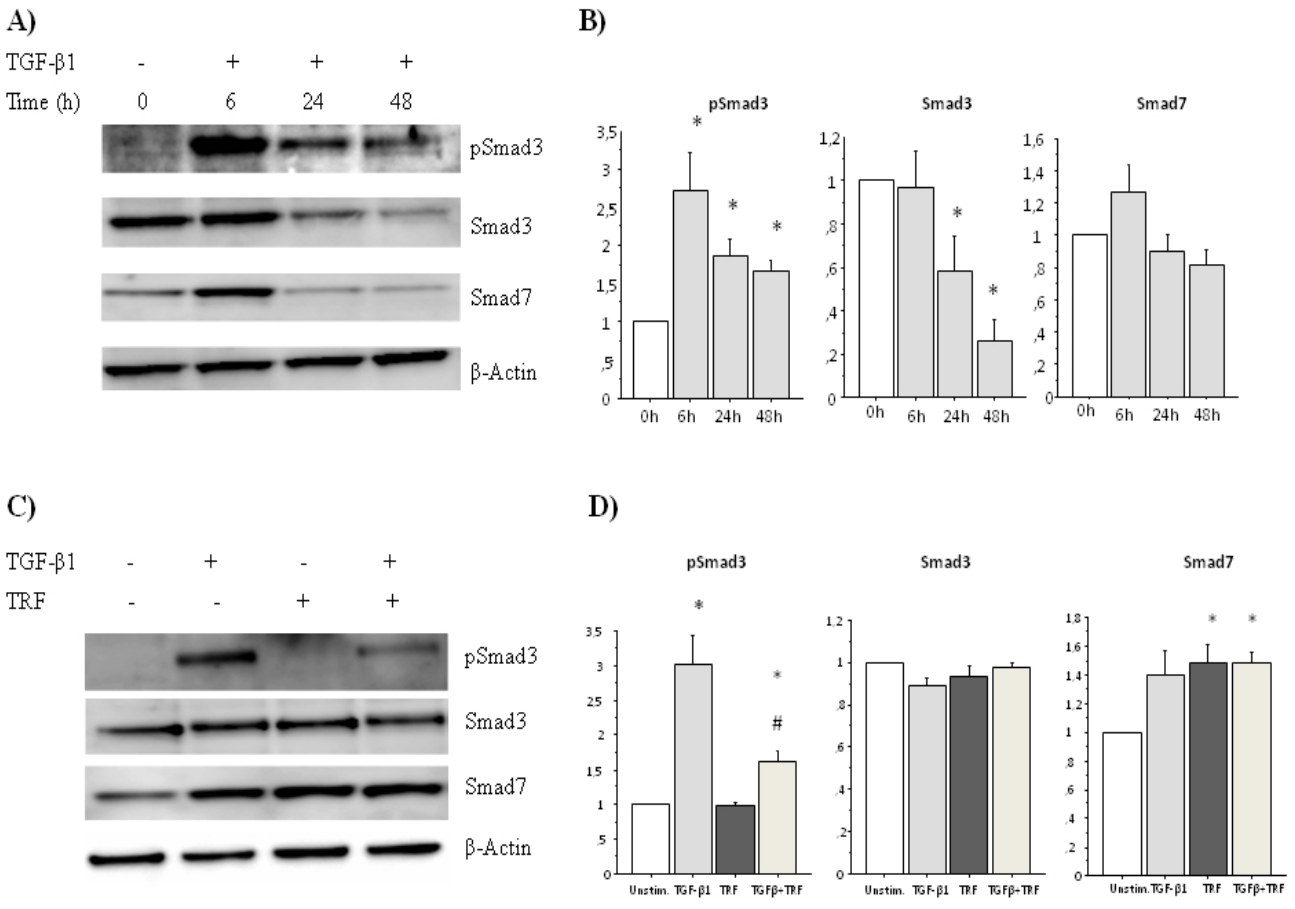
Figure 3. Effect of transforming growth factor-β1 (TGF-β1) on tissue inhibitor of metalloproteinases-1 (TIMP-1) and matrix metalloproteinase-3 (MMP-3) production by human intestinal fibroblasts (HIF). HIF were stimulated with 20 μM of TRF for 24 hours and TGF-β1 for 48 hours. (A) Representative immunoblot of TIMP-1 and MMP-3

production by HIF upon TGF-β1 stimulation. (B) Quantification of TIMP-1 production by HIF normalized by β-actin as determined by densitometric analysis of each immunoblot. (C) Quantification of MMP-3 production by HIF normalized by β-actin as determined by densitometric analysis of each immunoblot. Data are expressed as mean ± SEM; $n \geq 3$. *$P < 0.05$ vs. unstimulated cells, #$P < 0.05$ vs. TGF-β1.

Figure 4. Effect of tocotrienol rich fraction (TRF) on transforming growth factor-β1 (TGF-β1) mediated production of procollagen type 1 and type 3 by human intestinal fibroblasts (HIF). HIF were treated with TRF and TGF-β1 for 48 hours. (A) Representative immunoblot of procollagen type 1 and type 3 production by HIF upon TRF and TGF-β1. (B) Quantification of procollagen type 1 production by HIF normalized by β-actin as determined by densitometric analysis of each immunoblot. (C) Quantification of procollagen type 3 production normalized by β-actin as determined by densitometric analysis of each immunoblot. Data are expressed as mean ± SEM; $n \geq 3$. *$P < 0.05$ vs. unstimulated cells, **$P < 0.01$ vs. unstimulated cells, ***$P < 0.001$ vs. unstimulated cells, #$P < 0.05$ vs. TGF-β1.

Figure 5. Differential effect of transforming growth factor-β1 (TGF-β1) on procollagen type 1 production in Crohn's disease (CD) and control human intestinal fibroblasts (HIF). HIF were treated with TGF-β1 for different periods of time (A) and with TRF and TGF-β1 for 48 hous (B). (A) Quantification of procollagen type 1 production upon TGF-β1 stimulation by CD and control HIF. (B) Quantification of procollagen type 1 production showing the effect of TRF on TGF-β1-induced synthesis of procollagen type 1 in CD and control HIF. Data are expressed as mean ± SEM; $n \geq 3$. §$P < 0.05$ vs. normal HIF, *$P < 0.05$ vs. unstimulated cells , #$P < 0.05$ vs. TGF-β1.

Figure 1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Smad3
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β-Actin
B)
pSmad3
Smad3
Smad7
0h 6h 24h 48h
C)
TGF-β1 - + - +
TRF - - + +
pSmad3
Smad3
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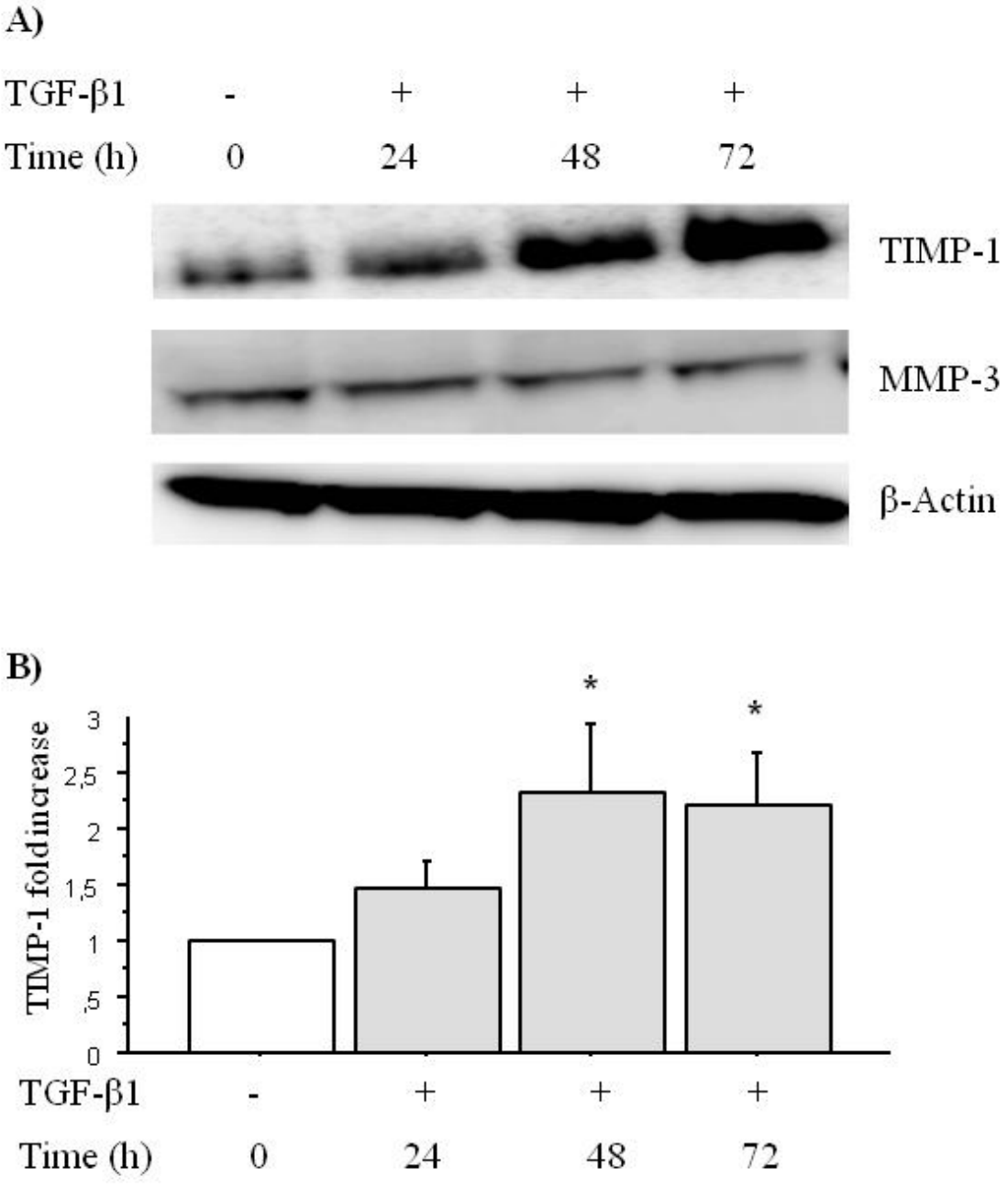
D)
pSmad3
Smad3
Smad7
Unstim. TGF-β1 TRF TGFβ+TRF

Figure 2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Time (h) 0 24 48 72
TIMP-1
MMP-3
β-Actin
B)
TIMP-1 foldincrease
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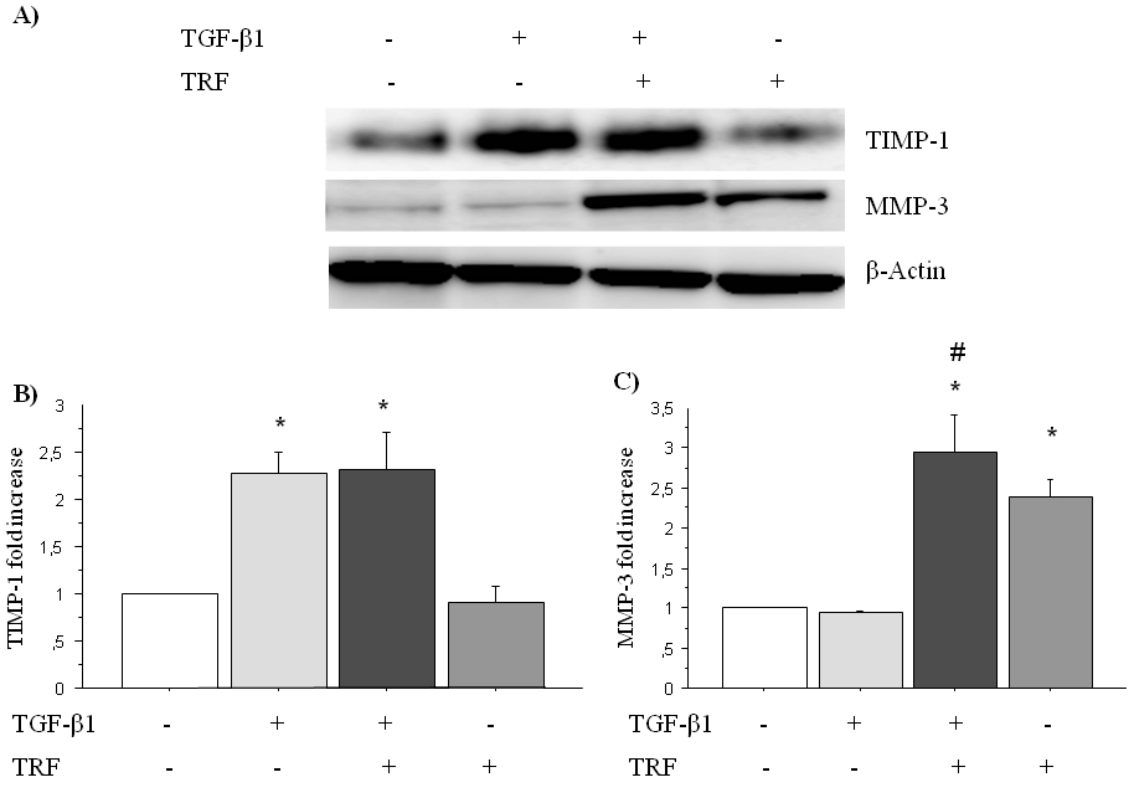
TGF-β1 - + + +
Time (h) 0 24 48 72

Figure 3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TIMP-1 fold increase
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MMP-3 fold increase
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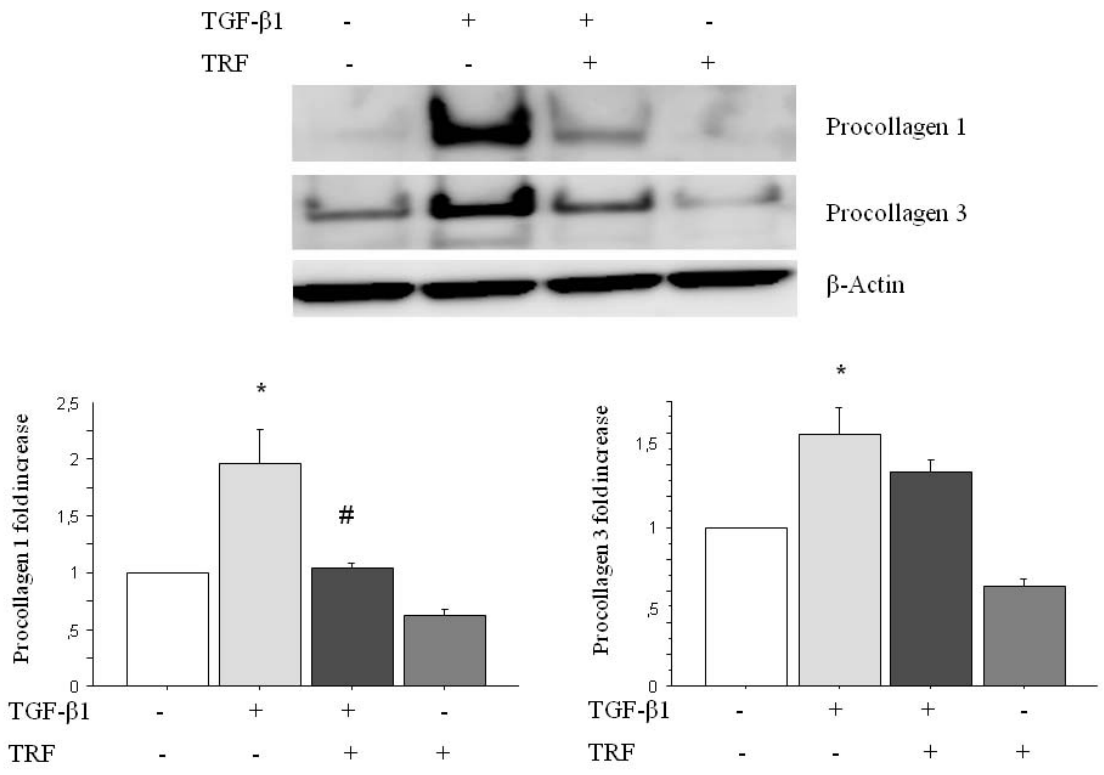
TGF-β1 - + + -
TRF - - + +

Figure 4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TGF-β1 - + + -
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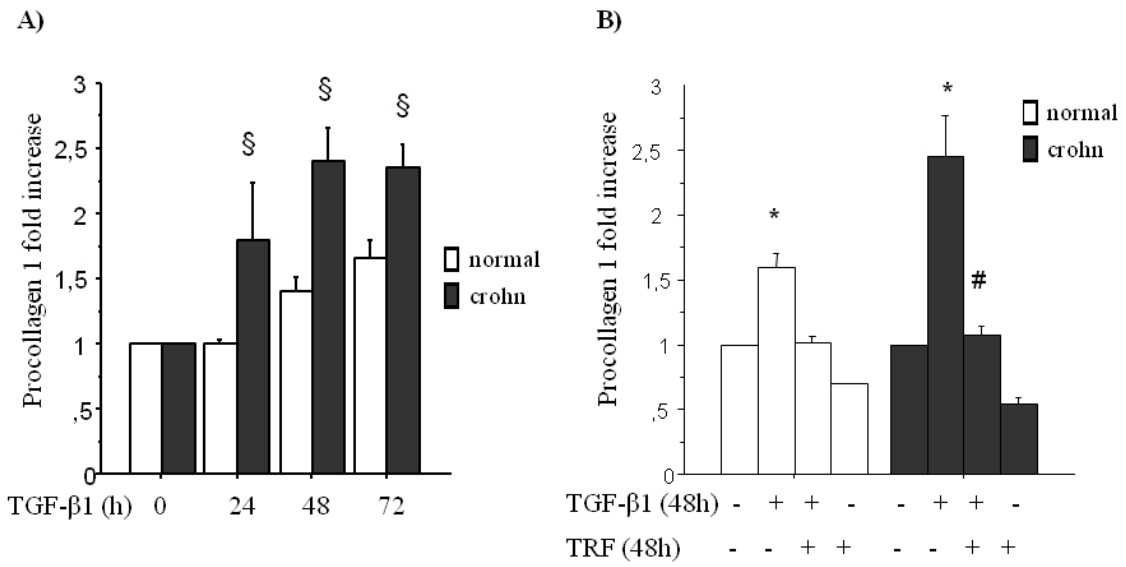
TRF - - + +

Figure 5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Procollagen 1 fold increase
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crohn
TGF-β1 (h) 0 24 48 72
B)
Procollagen 1 fold increase
3
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TGF-β1 (48h) - + + - - + + -
TRF (48h) - - + + - - + +

3) Tercer treball

Treatment of intestinal fibrosis with tocotrienols in an optimized rat model

Com ja hem demostrat en els dos anteriors treballs, la FRT té un potencial antifibrogènic “in vitro” en FIH. L’objectiu d’aquest nou treball és avaluar la utilitat de la FRT “in vivo” com a tractament antifibrogènic en un model optimitzat de fibrosi intestinal en la rata basat en l’administració repetida de TNBS.

La optimització del model ha permés determinar que la dosi de 10mg/setmana de TNBS és la dosi que indueix un major grau de fibrosi intestinal en la rata i, a més, que aquesta fibrosi és fàcilment reproduïble.

Es van testar 3 dosis diferents de FRT administrat de forma intragàstrica per tal d’esbrinar si la FRT és capaç de revertir o prevenir la fibrosi causada per l’administració de TNBS. Cap de les dosis de FRT va disminuir l’àrea de teixit fibròtic a les tres setmanes de tractament. Tot i això, el tractament amb la dosi més alta de FRT (150µl/dia) va reduir significativament la diarrea i sagnat rectal i la pèrdua de pes de l’animal. A més, també va reduir l’expressió de TNF-α i vimentina en el teixit afectat.

Treatment of intestinal fibrosis with tocotrienols in an optimized rat model

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Abstract

Background: Tocotrienols have potent antifibrogenic effects *in vitro* on human intestinal fibroblasts. The aim of this study was to evaluate the usefulness of tocotrienols to prevent the development of intestinal fibrosis in an optimized rat model. **Methods**: Sprague-Dawley rats were treated with several regimes of intrarectal TNBS. Once the optimal model was established, animals were treated with oral administration of 10, 50 or 150 μl/day of tocotrienol rich fraction (TRF) from palm oil, started 10 days prior to the first TNBS dose and continued for 3 weeks after the first TNBS dose. At this point animals were sacrificed and intestinal fibrosis was quantified in colonic preparations stained with Mason's trichromic, by means of a computer-assisted morphometric analysis. Colonic expression of collagen I and III, TNF-α and vimentin was measured by RT-PCR and TGF-β1 activation and MMP-3 and TIMP-1 production by Western-blot. **Results**: Treatment with 10 mg/week of intracolonic TNBS for 3 weeks was the best regime to induce intestinal fibrosis. After 1 week marked submucosal enlargement was due to inflammatory infiltrate and edema, which was substituted by fibrotic tissue at 3 weeks. None of the TRF doses was able to reduce the fibrotic tissue area. However, treatment with 150 μl of TRF significantly ($p<0.05$) reduced diarrhea, rectal bleeding and animal weight loss, as well as colonic TNF-α and vimentin expression. **Conclusions**: TRF did not prevent intestinal fibrosis in an optimized rat model. However, treatment with TRF had anti-inflammatory effects, decreasing some of the clinical hallmarks of TNBS-induced colitis. We therefore can't rule out the possibility that treatment with TRF for longer periods of time might improve intestinal fibrosis.

Introduction

Crohn's disease is a very heterogeneous condition and more than one third of CD patients will develop a fibrostenosing phenotype, characterized by progressive narrowing of the intestinal lumen[1]. In these patients abnormal bowel fibrogenesis is due to chronic transmural inflammation and impaired wound healing, which result in massive fibroblast proliferation and an excessive deposition of ECM in the bowel wall. Ultimately, abnormal contraction of the ECM will also contribute to the tissue distortion and intestinal obstruction.

No medical treatment for bowel fibrosis has become available to date, in spite of the remarkable success of the new, anti-inflammatory therapies recently developed for inflammatory bowel disease (IBD)[2]. Due to the lack of medical therapies for bowel fibrosis most CD patients with a stenosing phenotype will require surgical resection of the involved bowel segment, either once or often more times during their live.

Relatively minor progress has been done in our knowledge of the molecular mechanisms that lead to bowel fibrosis, as compared to liver, lung, kidney or skin fibrosis[3]. Animal models have greatly advanced our understanding of the mechanisms of gut inflammation. Such models, however, have focused almost exclusively on the immune-mediated mucosal inflammation with little attention to chronic disease and intestinal fibrosis.

The hapten 2, 4, 6-trinitrobenzenesulfonic acid (TNBS), administered as an enema, has been widely used to study colonic inflammation[4, 5]. This model, maintained for 7-10 days, is widely used to investigate immune events underlying the acute inflammatory responses in the gut[6, 7]. More recently repeated intracolonic TNBS administration has been shown to induce colonic fibrosis in mice[8]. However the effects of TNBS are highly variable and mice strain dependent.

A variety of natural dietary constituents, including vitamin E, have attracted the researcher's attention for their health benefits and harmless consumption profile. In nature, eight substances have been found to have vitamin E activity: α-, β-, γ- and δ-tocopherol; and α-, β-, γ- and δ-tocotrienol. To date most efforts had been devoted to α-tocopherol, due to its abundance in the human body and potent antioxidant activity. However, dietary tocotrienols are well absorbed, easily distributed throughout the body tissues and could provide

greater health benefit than α-tocopherol, due to their antiproliferative, neuroprotective, and cholesterol-lowering properties. The suppressive effects of tocotrienols on tumour growth are attributed to their ability to induce both cell cycle arrest and apoptosis in transformed cells[9-11].

Two previous studies from our group demonstrated that tocotrienols can induce apoptosis and autophagy in rat pancreatic fibroblasts[12] and in human intestinal fibroblasts[13], in vitro, suggesting a potential role of this compound in anti-fibrotic therapy.

The purpose of the present study was to evaluate the usefulness of tocotrienols to prevent the development of fibrosis in an optimized model of TNBS-induced fibrosis in the rat.

Materials and Methods

Reagents and antibodies

Cell culture flasks and clusters were from Corning (New York, NY). Tocotrienol Rich Fraction (TRF) from palm oil (Tocomin 50®, Carotech, Wendover, UK) was kindly provided by Carotech Ltd (Wendover, UK). High capacity cDNA reverse transcription kit, Taqman fast universal master mix, Col1a1, Col3a1, TNF-α and Vimentin Taqman probes and Rat actin beta probe were from Applied Biosystems (Carlsbad, CA). Rabbit anti-β-actin and TNBS were from Sigma-Aldrich (St. Louis, MO). Monoclonal TGF-β1antibody was from R&D Systems (Minneapolis, MN). Invitrolon polyvinylidene difluoride (PVDF) membranes and NuPage gels were from Invitrogen (Carlsbad, CA). Rabbit matrix metalloproteinase-3 (MMP-3) antibody and horseradish peroxidase (HRP)-conjugated antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). TACS 2-TdT-Fluor in situ apoptosis detection kit was from Trevigen (Gaithersburg, MD). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animals

Male Sprague Dawley rats weighing 300g were purchased from Charles River Laboratories Inc. (Wilmington, MA). Rats were housed and fed standard chow and tap water ad libitum throughout the study following protocols approved by the Animal Care and Use Committee at University of Barcelona School of Medicine.

Induction of colonic fibrosis. Time-course study of intestinal fibrosis development

Rats were lightly anesthetized by placing in a glass chamber containing isoflurane and randomized into control and treatment groups. In order to establish which was the optimal TNBS dose, two treatment groups were defined, to receive 3 doses of 5 mg or 10 mg of TNBS, respectively. 1 mL of TNBS (either 5mg or 10mg TNBS/enema) in 45% ethanol enema was administered. Each rat received 1 enema of TNBS on a

weekly basis for 3 weeks. The tip was inserted to a depth of 8 cm from the anal ring, the enema was slowly administered and the animal was held by the tail in a vertical position, head down, for 30 seconds to allow uniform distribution of the TNBS mixture. All animals were examined twice a week for signs of colitis including weight loss, diarrhea, rectal bleeding and prolapse.

Once the optimal dose of TNBS was established, and to better understand the process of intestinal fibrosis development, rats were sacrificed for histology and sample collection at day 1, 3, 7, 14 and 21 after the first TNBS enema administration. Rats sacrificed at day 1, 3 and 7 had received 1 TNBS enema. Rats sacrificed at day 14 had received a total of 2 TNBS enemas and rats sacrificed at day 21 had received a total of 3 TNBS enemas.

Study design

Sprague Dawley rats were randomized into 3 groups of tocotrienol treated animals and 2 groups of control animals. The effects of 3 doses of the tocotrienol rich compound Tocomin 50® were evaluated in this study: 10 µl, 50 µl and 150 µl. In all cases Tocomin 50® was dissolved in superolein to reach a final volume of 500 µl. Tocotrienol-treated animals received daily intragastric Tocomin 50® (10, 50 or 150 µl) for a period of 31 days. Colonic fibrosis was induced by means of 3 doses of 10 mg of TNBS intrarectaly administered at days 10, 17 and 24. Rats were sacrificed and colonic samples collected at day 31. Two control groups were planned. One received intragastric 500 µl tap water for a period of 31 days and intrarectal saline enema on days 10, 17 and 24 (no fibrosis group), the other received intragastric tap water and intrarectal TNBS on days 10, 17 and 24 (untreated fibrosis group).

Tissue processing

The colons were removed intact from the anus to the ileocecal junction; the length was measured, cleaned and weighed. At macroscopic examination, the distal 5 to 7 cm of the TNBS-treated colons were indurated, edematous, thickened, with evidence of colonic fibrosis. Sections were taken from these involved regions for

the following experiments: (1) formalin fixation and histological examination; (2) total RNA isolation; (3) total protein extraction.

Serial paraffin sections of the colon were stained with H&E and Mason's trichromic to detect connective tissue. Histological preparations were visualized under an inverted microscope Nikon Eclipse Ti-S and submucosal area was quantified relative to total transversal area of colonic tissue with NIS-Elements Br Nikon Software.

mRNA assessment by RT-PCR

Total RNA was isolated from full-thickness colonic tissue with RNeasy Kit, Qiagen (Valencia, CA). 500 ng aliquots of total RNA were reverse transcribed to assay for Col 1a1, Col 3a1, TNF-α and vimentin expression by real-time polymerase chain reaction with 7500 Fast Real-Time PCR System, Applied Biosystems (Carlsbad, CA). Results were standardized to β-actin.

Preparation of whole tissue lysates for protein detection

Tissue lysates for protein detection were prepared from full-thickness colonic tissue with RIPA buffer with Complete Protease Inhibitor Cocktail. Tissue lysates were centrifuged at 8500 rpm for 5 min and supernatants obtained for TGF-β1 and MMP-3 measurement.

Westerm blot analysis

Whole tissue lysates were separated in Nu-PAGE 4%-12% Bis-Tris gels with MOPS-SDS running buffer and transferred to PVDF membranes. The membranes were blocked and probed with antibodies against TGF-β1 (1/1000), MMP-3 (1/200) and β-actin (1/5000) with an overnight incubation at 4ºC and with HRP-conjugated antibodies for 1 hour at room temperature. Blots were developed by using the Pierce SuperSignal WestFemto Maximum Sensitivity Substrate (Pierce, Rockford, IL) and bands were visualized using a Fujifilm Image Reader LAS-3000 phosphoimager (Fujifilm Photo Film Co., Tokyo, Japan). Quantification of protein

expression was determined by densitometry of digitalized images by using Image Gauge V4.0 (Fujifilm Photo Film Co., Tokyo, Japan).

TUNEL evaluations

TUNEL experiments were performed following manufacturer's instructions. Briefly, tissue slides were deparaffinizated and incubated with Proteinase K for 30 minutes at room temperature, washed and incubated 1 hour at 37ºC with labeling reaction mix in a humidity chamber. Tissue slides were incubated with Strep-Fluor for 20 minutes at room temperature, immersed in fluorescence mounting medium and observed under a Nikon fluorescence microscope. Some samples were pre-treated with TACS-Nuclease to generate positive controls of the staining method.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test for non-parametric data, and results are expressed as mean ± SEM. A P value <0.05 was considered significant.

Results

Effects of different doses of TNBS on colonic fibrosis

Since to date the rat model of TNBS-induced colonic fibrosis has only been based on a single intrarectal administration of TNBS, with variable results, we first aimed at investigating a) the effects of repeated TNBS administration on colonic fibrosis in rats, and b) which is the optimal dose of TNBS. We found that the administration of 3 doses of 10 mg of TNBS, at days 0, 7 and 14 followed by animal study at day 21, resulted in a much more intense colonic fibrosis compared to the administration of 3 doses of 5 mg of TNBS at the same time points, as shown in supplementary figures 1 and 2.

Development of TNBS-induced colonic fibrosis. Time-course study

Having defined the optimal dose of TNBS, we next aimed at studying the development of intestinal fibrosis over time. TNBS-treated rats showed a decreased body weight compared to control rats. This reduction in the growth ratio started immediately after the first administration of TNBS and was observed during all the treatment period reaching statistical significance from the second TNBS dose and until the end of treatment (Fig 1A). Rectal bleeding and diarrhoea were observed 2 days after TNBS administration (Figure 1B). Rectal prolapse and periorbital exudates were not observed. At macroscopic examination, the distal 5 to 7 cm of the TNBS-treated animals were indurated, edematous and thickened. No inflammatory changes were observed in the control animals. As expected, an increase in colon weight, a reduction in colon length and an increase in colon thickness were observed as soon as three days after the first TNBS administration and later on (Fig 1C, D and E).

The submucosal surface was dramatically increased three days after TNBS administration compared to control group. This initial expansion of the colonic submucose was characterized by an inflammatory infiltrate and edema, which was progressively substituted by cellular and extracellular matrix components

(Fig. 2). The switch from inflammatory to fibrotic changes resulted in a moderate decrease of the submucosal area over time (Fig. 2J).

Treatment with TNBS for one and two weeks stimulated collagen I and III colonic expression (Fig. 3A and B). In addition, expression of vimentin, a marker of fibroblasts and myofibroblasts (Fig. 3D) and TNF-α (Fig 3C) was also increased at the RNA level by TNBS treatment. Although total levels of TGF-β1 protein were not modified by TNBS treatment at any time, an increase in active TGF-β1 was observed two and three weeks after TNBS treatment (Fig. 4A and B). On the contrary, MMP-3 production was strongly up-regulated. After three days of the first TNBS administration MMP-3 levels gradually returned to normal during the study period being similar to baseline at the end of it (Fig. 4 C and D).

Effects of TRF on TNBS-induced colonic inflammation and fibrosis

The use of high doses (50 μl and 150 μl) of TRF, attenuated the TNBS-induce decrease in body weight. This effect was observed after the second TNBS administration and reached statistical significance in the TRF 150 μl group which restored the growth ratio at the end of treatment (Fig. 5A). Similarly, the DAI score was also significantly reduced in the TRF 150 μl group as compared to the control group (Fig. 5B). On the contrary, TRF had no effect on colon length, weight or thickness at any dose (Fig. 5C, D and E).

Treatment with TRF did not prevent the TNBS-induced increase in submucose area at any of the doses tested (Fig. 6). Treatment with TRF had no influence on collagen I and III expression either (Fig. 7A and B). However, the dose of 150 μl of TRF was able to markedly reduce the colonic expression of TNF-α and vimentin (Fig. 7C and D). Levels of activated TGF-β1 remained unchanged after treatment with TRF (Fig. 8A and B).

Since the induction of fibroblast apoptosis is one of the key anti-fibrogenic effects displayed by tocotrienols *in vitro*, we also aimed at determining the extent of apoptosis caused by TRF treatment on colonic submucosal fibroblasts. After three weeks of TNBS-induced fibrosis a considerable amount of apoptotic cells

could be seen in areas of fibrosis, most of the apoptotic cells were located in the submucosal layer. However, treatment with TRF did not change the number or localization of these apoptotic cells (Fig. 9).

Discussion

In 2003, Lawrance et al.[8] reported that one can establish a chronic hapten-induced colitis in BALB/c mice by the repeated intrarectal administration of TNBS and that such colitis was ultimately accompanied by the occurrence of gastrointestinal fibrosis. Since then several groups have used this model to study intestinal inflammatory and fibrotic events[14,15]. In this study, we took the idea of repeated TNBS administration to induce gastrointestinal fibrosis in the mouse and we adapted this model to a rat model of fibrosis. We characterized the clinical, colonic macroscopic and microscopic and molecular changes that characterizes various stages of TNBS administration and we tested the ability of intragastric treatment with tocotrienol rich fraction to reduce TNBS induced intestinal inflammation and fibrosis.

The course of chronic TNBS colitis can be divided into three distinct phases. The first phase, lasting from day 0 until approximately day 7, is characterized by an acute inflammation leading to extensive tissue damage and inflammatory cell infiltration; there is a reduction in body weight increase. Colonic changes in this stage include gain in total colon weight, a small reduction in colon length and consequently an increase in the thickness of the colon wall.

TNF-α is known to up regulate MMP-1 and MMP-3 production by intestinal fibroblasts[16-18]. According to these observations in our model of TNBS administration TNF-α colonic mRNA levels are elevated and there is an important up regulation of MMP-3 protein during the first phase of TNBS induced colitis.

The next phase lasting from about day 7 through day 14 is marked by continued but nonprogressive inflammation that allows the rats to maintain but not increase body weight. During this phase, submucose inflammatory infiltrate is progressively replaced by cellular and matrix components. Expression of collagens is elevated, and so it is expression of TNF-α and vimentin. Vimentin is a marker of mesenchymal derived cells[19] and has been implicated in epithelial to mesenchymal transition (EMT), a process in which a polarized epithelial cell undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components[20-22].

The third phase of chronic intestinal inflammation induced by TNBS lasting from day 14 to 21 is characterized by a slight recovery in body weight. Inflammatory infiltrate in colonic submucose is completely substituted by fibrotic tissue and there is a marked activation of the profibrotic cytokine TGF-β[23]. TGF-β has been described to suppress MMP-3 and MMP-9 expression[24,25], according to these findings we observed a marked reduction in colonic MMP-3 levels in parallel to activation of TGF-β.

Two previous studies from our group demonstrated that tocotrienols can induce apoptosis and autophagy in rat pancreatic fibroblasts[12] and in human intestinal fibroblasts[13], in vitro, suggesting a possible potential of this compound in antifibrotic therapy. We investigated the effectiveness of TRF to reduce fibrogenic connective tissue changes associated with chronic inflammation. TNBS-treated rats that were given TRF, macroscopic signs of the disease, namely diarrhea and significant weight loss were reduced in a dose dependent manner. Thricrome staining of colonic cross-sections did not show a reduction of fibrogenic tissue architecture but expression of TNF-α and vimentin was significantly reduced at higher TRF doses.

The fact that high doses of TRF are able to reduce disease activity index and TNF-α expression in TNBS treated rats is supported by previous findings of other groups in which an anti-inflammatory effect of tocotrienols is demonstrated. Namely, tocotrienols suppress the expression of inflammatory cytokines, such as TNF-α, IL-4, and IL-8, and down-regulation of NF-κB in LPS-induced human monocytic cells[26]. Furthermore, pre-treatment with novel tocotrienols has been reported to reduce the induction of TNF-α in response to bacterial LPS in mice[27].

As mentioned above, vimentin has been implicated in EMT, a process contributing to tissue fibrosis[28-30]. The vimentin gene (VIM) has a promoter with multiple elements responsible for its transcriptional regulation. An NF-κB binding site has been implicated in growth factor responsiveness, and in the induction of VIM mRNA in response to expression of the Human T-lymphotrophic virus-1 (HTLV-1) Tax protein. Tax-induced activation of NF-κB leads to NF-κB binding to an element on the VIM promoter[31-33].

According to these observations, the reduction of TNF-α and vimentin mRNA levels observed after treatment of TNBS colitic rats with TRF could be explained by inhibition of NF-κB activation.

It is generally accepted that apoptosis of mesenchymal cells is responsible for spontaneous resolution of fibrosis in several tissues. In that regard, it has been demonstrated that apoptosis of hepatic stellate cells is the main process leading to tissue restitution after liver cirrhosis[34,35]. In our model of TNBS-induced colonic fibrosis we could observe the presence of apoptotic cells in fibrotic areas of the colon. This could be a spontaneous mechanism leading to normal tissue restitution. Our group has demonstrated that TRF is able to set up a full apoptotic response in pancreatic stellate cells and human intestinal fibroblasts *in vitro*[12,13]. Treatment of TNBS fibrotic rats with TRF could accelerate this restitution process by inducing fibroblasts apoptosis, however after 30 days of TRF treatment we could not observe a significant increase in the number of apoptotic cells.

In conclusion, we developed a novel rat model of chronic immune-mediated inflammation and associated fibrosis of the colon displaying certain features of CD. Histologically, CD and our TNBS model show a full-thickness inflammation associated with increased ECM deposition and distortion of colonic tissue architecture. TRF treatment given prophyllactically restored body weight and reduced TNBS-induced inflammation but not fibrosis. Given the reduction in colonic vimentin expression observed upon TRF treatment we hypothesize that a longer treatment with TRF could be effective in reducing TNBS-associated fibrosis.

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Figure legends

Figure 1. Clinical features of chronic TNBS administration in Sprague Dawley. A) Gain in body weight as a percentage of starting weight. B) Diarrhea and rectal bleeding scores. C) Colon weight, D) Colon length and E) Ratio between colon weight and colon length. Data are expressed as mean ± SEM from at least 10 rats per group; *p<0.05 vs. control group, **p<0.01vs. control group, ***p<0.001 vs. control group.

Figure 2. Colonic histology of chronic TNBS administration. A) Control, saline-treated. B) 3 days after first administration of 10 mg of TNBS, C) and D) 3 days after first administration of 10 mg of TNBS showing inflammatory changes in the submucose, E) 1 week after first administration of 10 mg of TNBS, F) 2 weeks after the initiation of TNBS treatment, G) 3 weeks after the initiation of TNBS treatement, H) and I) 3 weeks after the initiation of TNBS treatment showing fibrotic changes in the submucose, J) submucose area quantified relative to total tranversal area of colonic section with NIS-Elements Br Nikon Software. Data are expressed as mean ± SEM from at least 10 rats per group; *p<0.05 vs. control group.

Figure 3. Colonic expression of A) collagen I, B) collagen III, C) TNF-α and D) vimentin at weekly time points during chronic TNBS administration in rat determined by RT-PCR. Data are expressed as mean ± SEM from at least 10 rats per group; *p<0.05 vs. control group, **p<0.01vs. control group, ***p<0.001 vs. control group.

Figure 4. TGF-β1 and MMP-3 colonic protein expression during the course of chronic TNBS colitis in Sprague Dawley rats, A) Representative western-blot showing activation of TGF- β1 in the late phase (week 2 and 3) of chronic TNBS administration. A total of 40 μg per lane were charged onto the gel, B) Quantification of activated versus total TGF- β1 normalized to β-actin, as determined by densitometric analysis of each immunoblot, C) Representative western-blot showing MMP-3 production in the acute phase of TNBS administration. A total of 50 μg per lane were charged onto the gel, D) Quantification of MMP-3 production normalized to β-actin, as determined by densitometric analysis of each immunoblot. Data are expressed as mean ± SEM from at least 10 rats per group; *p<0.05 vs. control group.

Figure 5. Clinical features of chronic TNBS administration in Sprague Dawley rats and effects of different doses of tocotrienols. A) Gain in body weight as a percentage of starting weight, B) Diarrhea and rectal bleeding scores, C) Colon weight, D) Colon length and E) Ratio between colon weight and colon length. Data are expressed as mean ± SEM from at least 10 rats per group; *$p<0.05$ vs. control group, ***$p<0.001$ vs. control group.

Figure 6. Colonic histology of chronic TNBS administration in Sprgue Dawley rats and effects of different doses of tocotrienols. A) Control, saline-treated. B) 3 weeks after first administration of 10 mg of TNBS showing fibrotic changes in the submucose, C) 3 weeks after first administration of 10 mg of TNBS with daily intragastric treatment with 150 µl of tocotrienols, D) submucose area quantified relative to total tranversal area of colonic section with NIS-Elements Br Nikon Software. Data are expressed as mean ± SEM from at least 10 rats per group; *$p<0.05$ vs. control group.

Figure 7. Colonic expression of A) collagen I, B) collagen III, C) TNF-α and D) vimentin 3 weeks after first administration of 10 mg of TNBS in rats and daily intragastric treatment with different doses of tocotrienols determined by RT-PCR. Data are expressed as mean ± SEM from at least 10 rats per group; *$p<0.05$ vs. control group, **$p<0.01$vs. control group, #$p<0.05$ vs. TNBS group.

Figure 8. TGF-β1 and MMP-3 colonic protein expression after 3 weeks of TNBS administration and dayly intragastric treatment with tocotrienols, A) Representative western-blot showing activation of TGF- β1. A total of 40 µg per lane were charged onto the gel, B) Quantification of activated versus total TGF- β1 normalized to β-actin, as determined by densitometric analysis of each immunoblot, C) Representative western-blot showing MMP-3 production. A total of 50 µg per lane were charged onto the gel, D) Quantification of MMP-3 production normalized to β-actin, as determined by densitometric analysis of each immunoblot. Data are expressed as mean ± SEM from at least 10 rats per group; ***$p<0.001$ vs. control group.

Figure 9. Detection of apoptotic cells in colonic tissue slides from control group, TNBS-treated group and TNBS+TRF 150µl treated group. A) The upper panel shows TACS-Nuclease pre-treated slides. B) and C) Visualization of apoptotic cells in the colonic submucose of TNBS and TNBS+TRF treated groups.

Figure 1

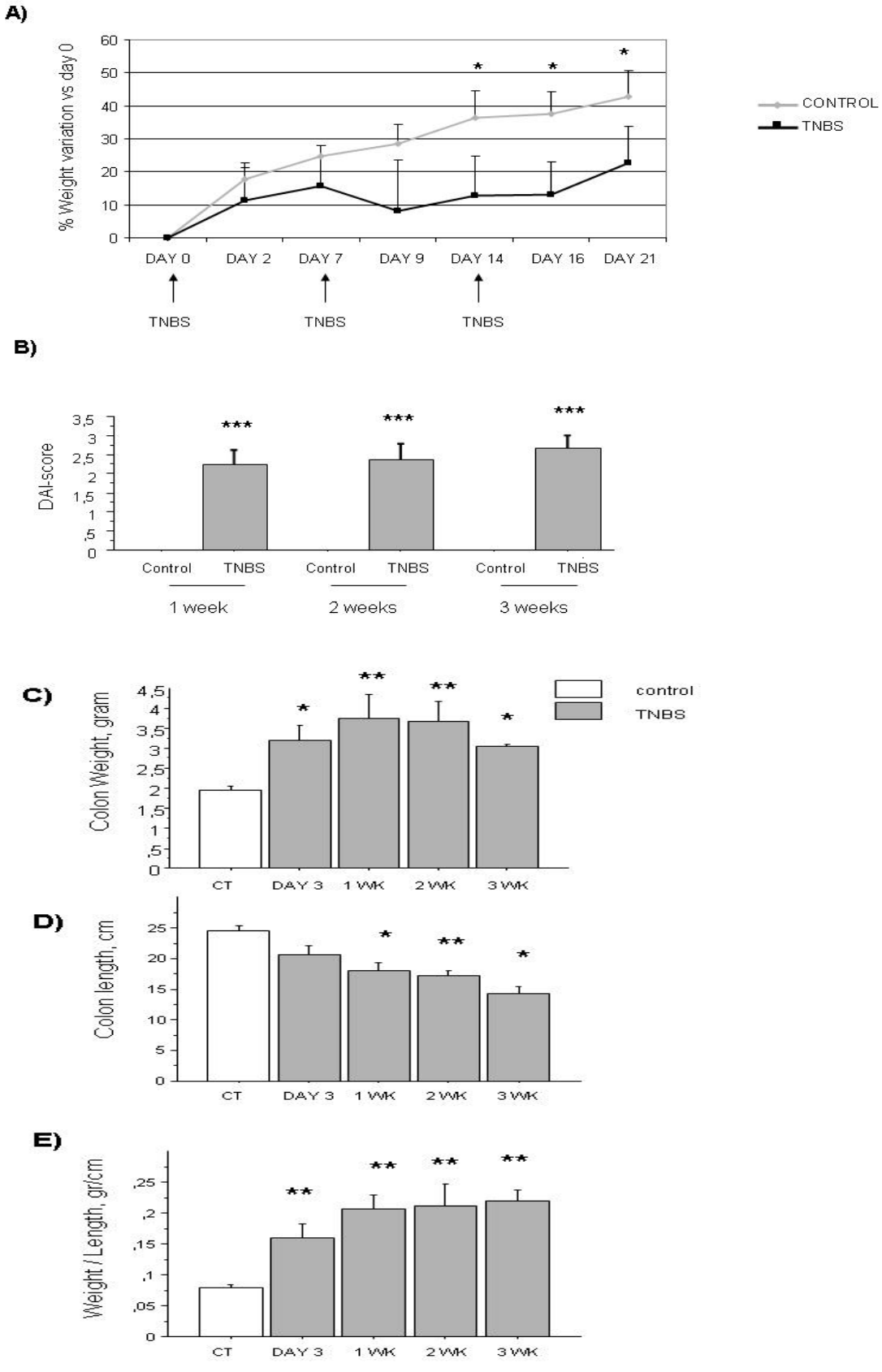
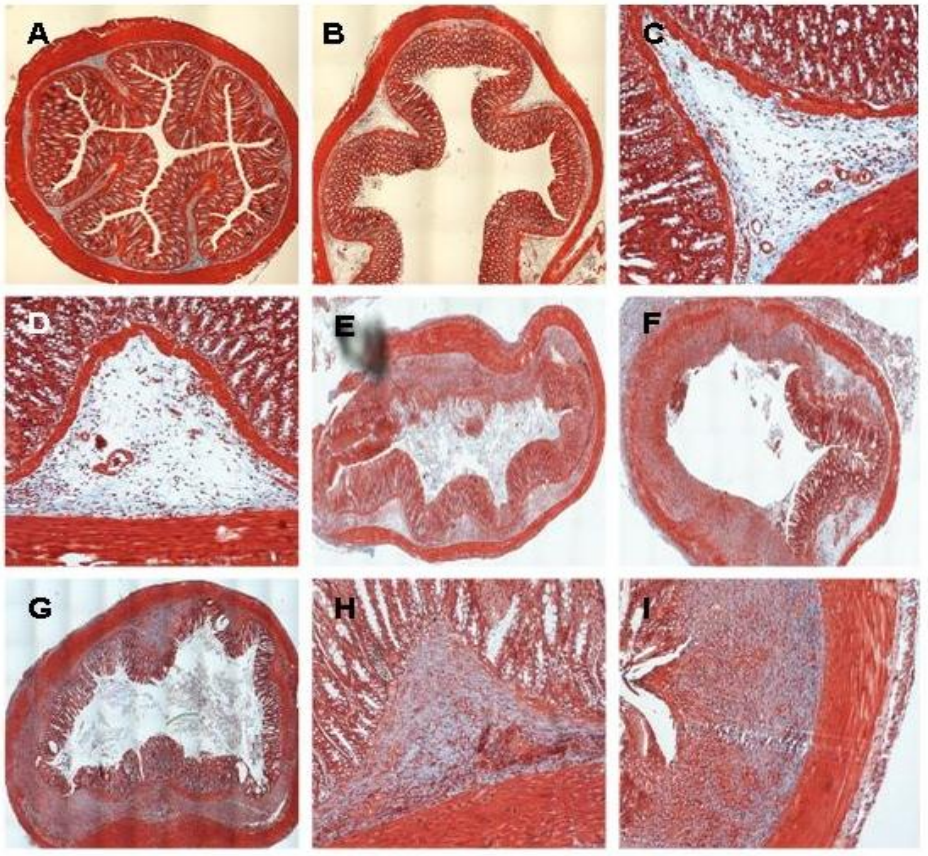

A)
% Weight variation vs day 0
CONTROL
TNBS
DAY 0
DAY 2
DAY 7
DAY 9
DAY 14
DAY 16
DAY 21
TNBS
B)
DAI-score
Control
TNBS
1 week
2 weeks
3 weeks
C)
Colon Weight, gram
control
TNBS
CT
DAY 3
1 WK
2 WK
3 WK
D)
Colon length, cm
E)
Weight / Length, gr/cm

Figure 2


A
B
C
D
E
F
G
H
I

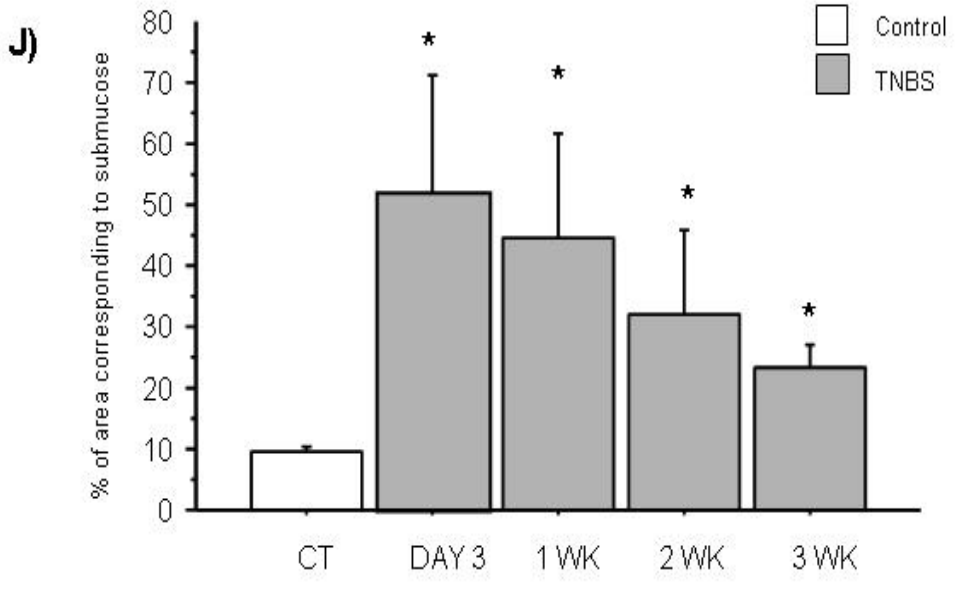

J)
% of area corresponding to submucose
80
70
60
50
40
30
20
10
0
Control
TNBS
*
*
*
*
CT
DAY 3
1 WK
2 WK
3 WK

Figure 3

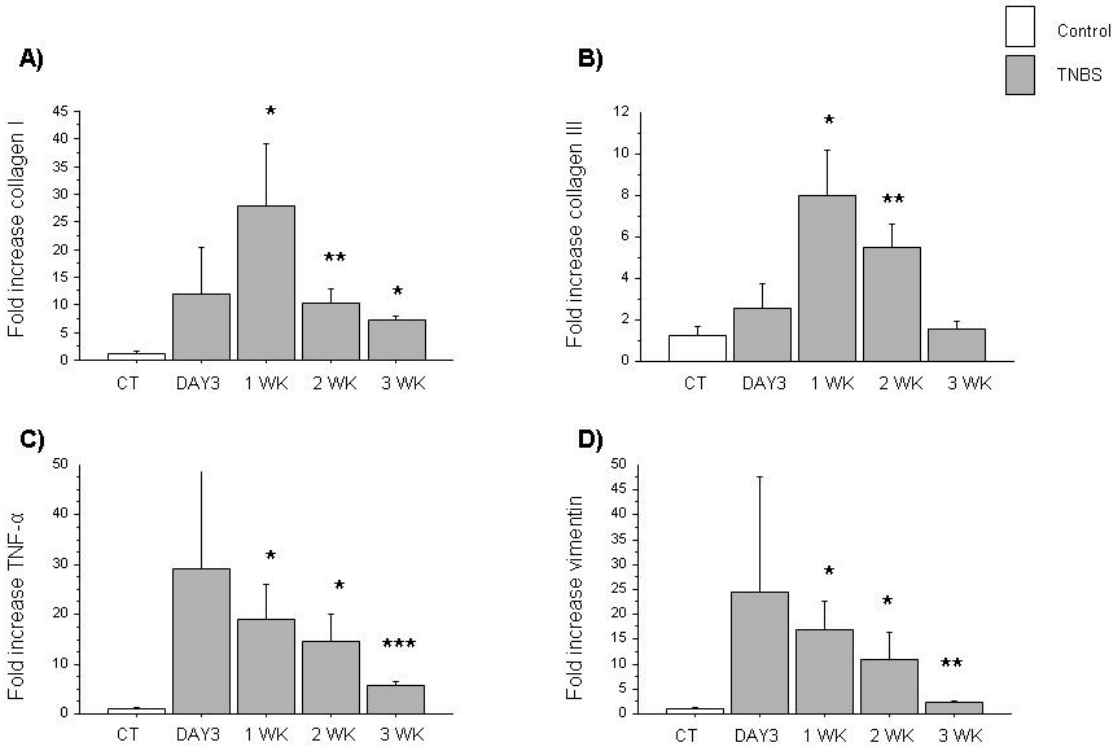

A)
B)
C)
D)
Control
TNBS
Fold increase collagen I
Fold increase collagen III
Fold increase TNF-α
Fold increase vimentin
CT
DAY3
1 WK
2 WK
3 WK
*
**
***

Figure 4

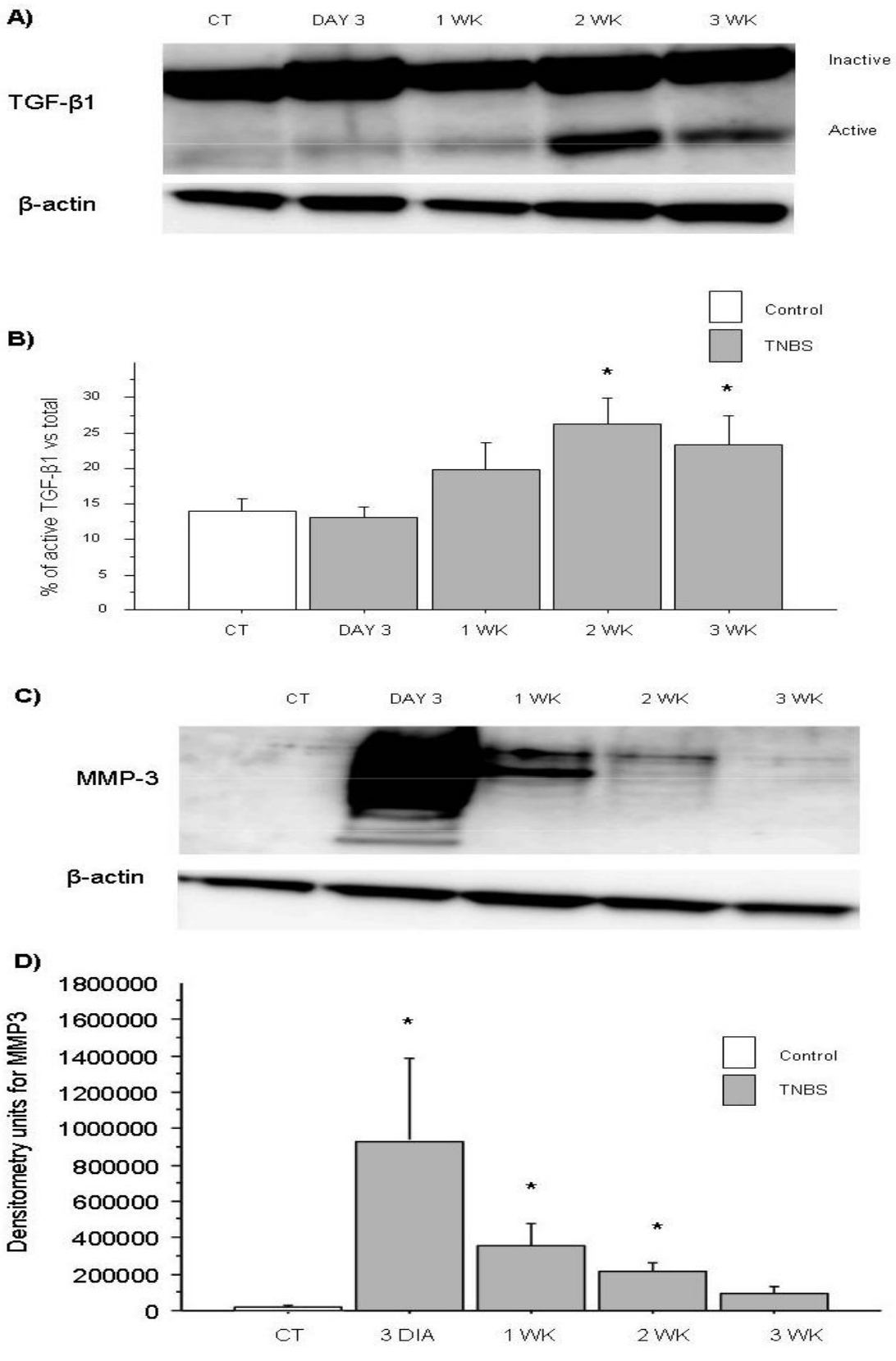
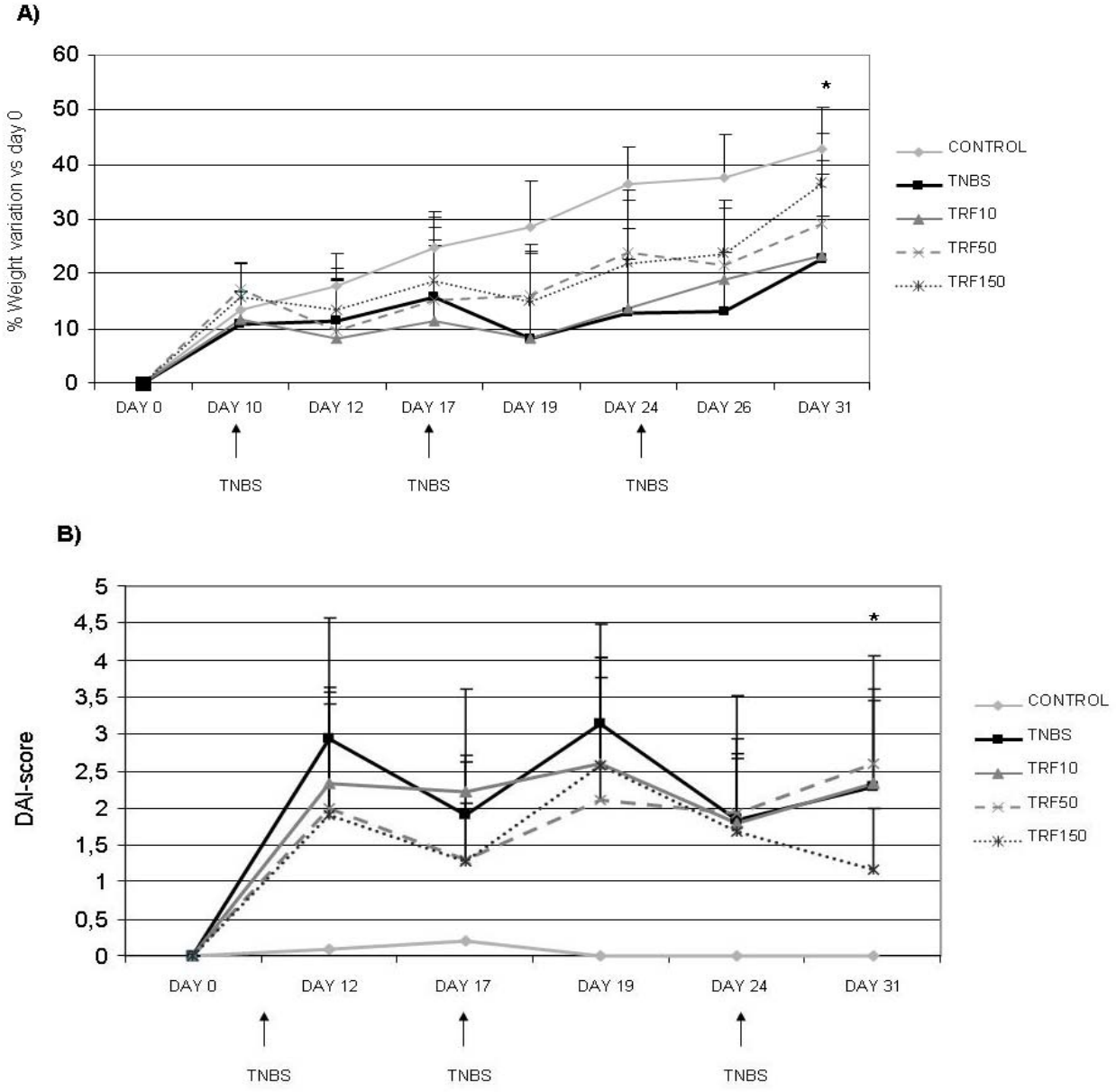

A)
CT
DAY 3
1 WK
2 WK
3 WK
TGF-β1
Inactive
Active
β-actin
B)
Control
TNBS
% of active TGF-β1 vs total
0
5
10
15
20
25
30
*
*
CT
DAY 3
1 WK
2 WK
3 WK
C)
CT
DAY 3
1 WK
2 WK
3 WK
MMP-3
β-actin
D)
Densitometry units for MMP3
0
200000
400000
600000
800000
1000000
1200000
1400000
1600000
1800000
*
*
*
Control
TNBS
CT
3 DIA
1 WK
2 WK
3 WK

Figure 5


A)
% Weight variation vs day 0
60
50
40
30
20
10
0
DAY 0
DAY 10
DAY 12
DAY 17
DAY 19
DAY 24
DAY 26
DAY 31
TNBS
TNBS
TNBS
*
CONTROL
TNBS
TRF10
TRF50
TRF150
B)
DAI-score
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
DAY 0
DAY 12
DAY 17
DAY 19
DAY 24
DAY 31
TNBS
TNBS
TNBS
*
CONTROL
TNBS
TRF10
TRF50
TRF150

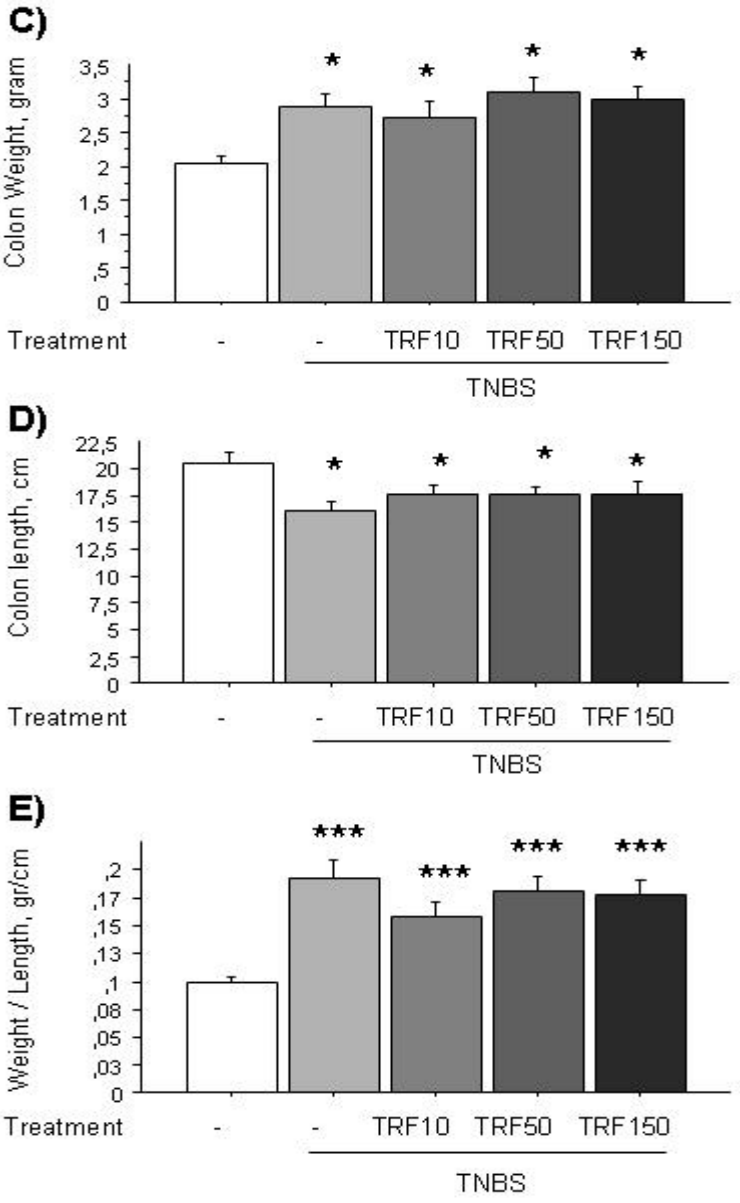
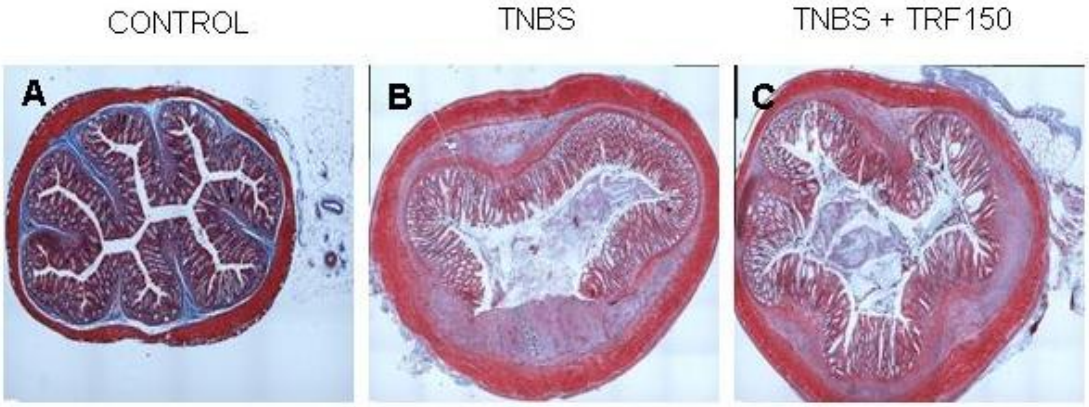

C)
Colon Weight, gram
3,5
3
2,5
2
1,5
1
,5
0
*
*
*
*
Treatment
-
-
TRF10
TRF50
TRF150
TNBS
D)
Colon length, cm
22,5
20
17,5
15
12,5
10
7,5
5
2,5
0
*
*
*
*
Treatment
-
-
TRF10
TRF50
TRF150
TNBS
E)
Weight / Length, gr/cm
,2
,17
,15
,13
,1
,08
,05
,03
0
***
***
***
***
Treatment
-
-
TRF10
TRF50
TRF150
TNBS

Figure 6


CONTROL
TNBS
TNBS + TRF150
A
B
C

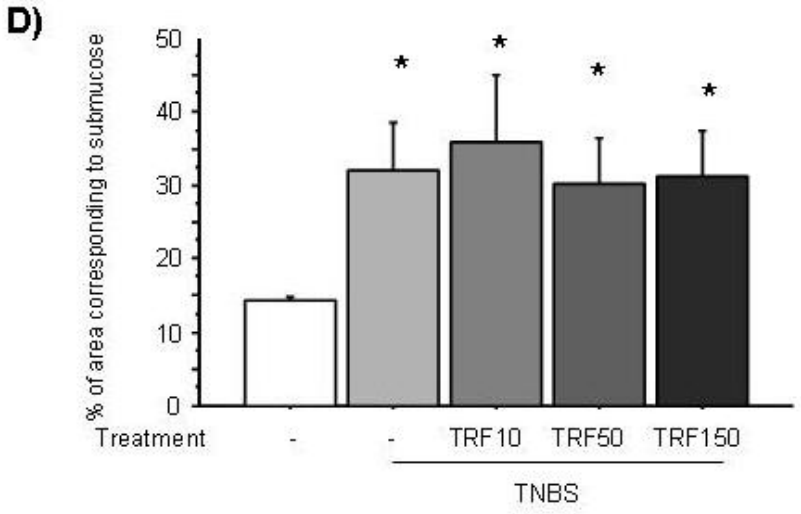

D)
% of area corresponding to submucose
50
40
30
20
10
0
Treatment
-
-
TRF10
TRF50
TRF150
TNBS

Figure 7

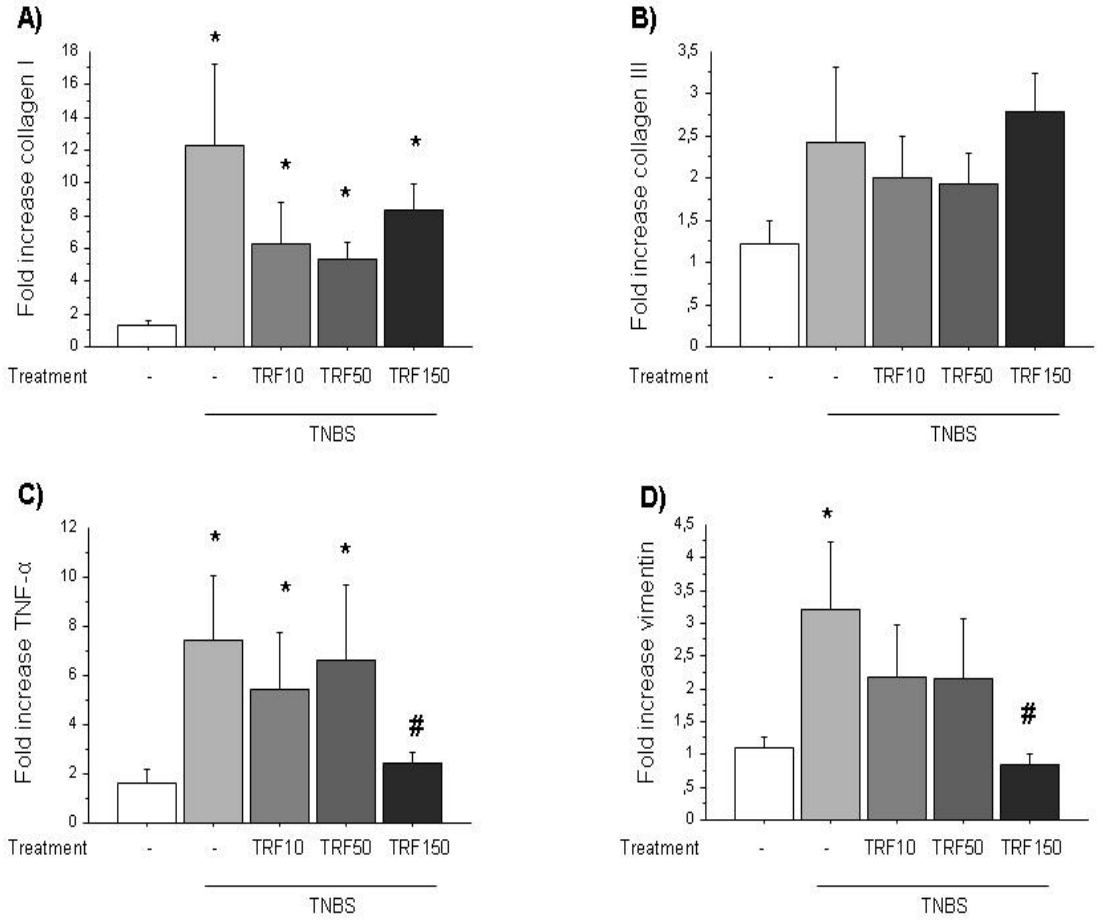

A)
Fold increase collagen I
18
16
14
12
10
8
6
4
2
0
*
*
*
*
Treatment
-
-
TRF10
TRF50
TRF150
TNBS
B)
Fold increase collagen III
3,5
3
2,5
2
1,5
1
,5
0
Treatment
-
-
TRF10
TRF50
TRF150
TNBS
C)
Fold increase TNF-α
12
10
8
6
4
2
0
*
*
*
#
Treatment
-
-
TRF10
TRF50
TRF150
TNBS
D)
Fold increase vimentin
4,5
4
3,5
3
2,5
2
1,5
1
,5
0
*
#
Treatment
-
-
TRF10
TRF50
TRF150
TNBS

Figure 8

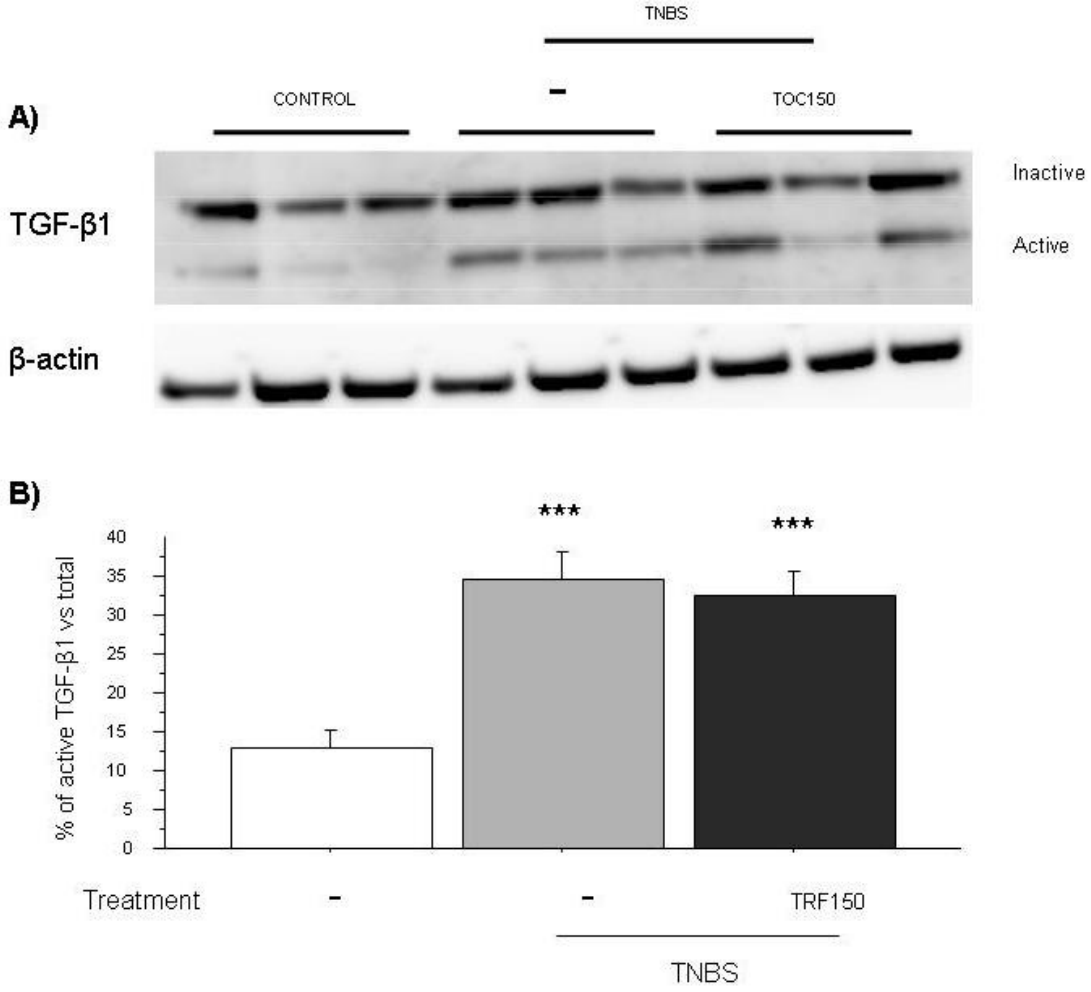
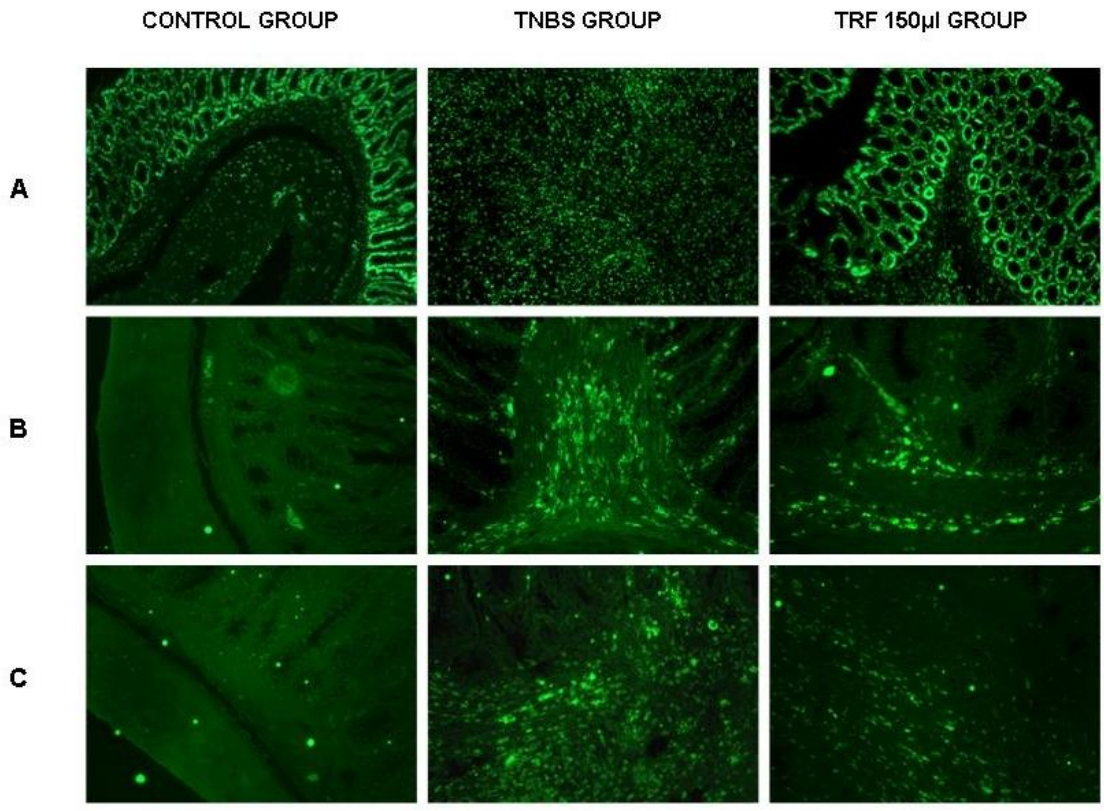

TNBS
A)
CONTROL
-
TOC150
TGF-β1
Inactive
Active
β-actin
B)
***
***
% of active TGF-β1 vs total
40
35
30
25
20
15
10
5
0
Treatment
-
-
TRF150
TNBS

Figure 9



DISCUSSIÓ

Els resultats del present Projecte de Tesi Doctoral demostren que la FRT té efectes múltiples en FIH en cultiu. Aquests efectes inclouen la inhibició en la proliferació cel·lular, la inducció de mort cel·lular programada mitjançant apoptosi i autofàgia i la reducció en la síntesi de MEC. Aquests efectes de la FRT sobre el funcionament cel·lular apunten a un potencial efecte antifibrogènic que podria ser útil en el tractament de la fibrosi intestinal en pacients amb MC.

En el patró fibroestenosant de la MC els fibroblasts intestinals augmenten en número contribuint al desenvolupament de la fibrosi. Un dels principals promotors de la proliferació d'aquestes cèl·lules és el bFGF[74,75]. En el present estudi la FRT ha demostrat tenir efectes antiproliferatius en FIH independentment de la procedència dels fibroblasts. La FRT redueix tant la proliferació basal com la induïda per bFGF en FIH en cultiu. Paradoxalment, en situació basal, sense bFGF, els tocotrienols inhibeixen la proliferació tant en cèl·lules control com en cèl·lules procedents d'àrees afectades. En canvi, quan s'afegeix bFGF al medi de cultiu, la FRT només és capaç de reduir la proliferació en cèl·lules aïllades de zones afectades de l'intestí però no en cèl·lules control. Aquest, és un resultat molt interessant ja que indica un efecte antiproliferatiu selectiu en cèl·lules procedents de MII.

Malauradament, durant la realització d'aquest Projecte de Tesi Doctoral, no hem pogut trobar una explicació satisfactòria al fet que la FRT inhibeixi la proliferació de manera selectiva quan les cèl·lules estan estimulades amb bFGF però no demostri cap tipus de selecció en situació basal. Sabem per altres estudis que els tocotrienols excerceixen els seus efectes en cèl·lules activades però no en cèl·lules quiescents[73]. Els fibroblasts control no es poden considerar cèl·lules inactives o quiescents, ja que el contacte amb una superfície plàstica, com és el material de cultiu sobre el qual es creixen aquestes cèl·lules, en provoca l'activació[76,77]. A més, amb la estimulació amb bFGF, els fibroblasts control demostren uns nivells de proliferació similar a fibroblasts aïllats de MII. Tot i això, és possible que les cèl·lules de MII conservin algunes característiques d'activació que les diferenciïn de les cèl·lules control i les faci més vulnerables a l'acció de la FRT.

Tal com ja hem comentat anteriorment, l'apoptosi de les cèl·lules efectores en el desenvolupament de la fibrosi sembla ser el principal mecanisme de la seva resolució. És sabut que en la MII hi ha una resistència cel·lular a l'apoptosi[78]. La FRT és capaç d'instaurar un procés complert d'apoptosi en els FIH en cultiu. A diferència del que passa amb la majoria d'agents proapoptòtics, la FRT produeix l'activació tant de la via extrínseca com de la via intrínseca de l'apoptosi ja que activa les caspases 8 i 9.

Sorprenentment, l'addició de ciclosporina A (CsA), un conegut inhibidor de la via intrínseca de l'apoptosi que actua bloquejant el porus mitocondrial[79], bloqueja completament el procés d'apoptosi induït per FRT. Això demostra que tot i l'activació de la caspasa 8 i per tant, de la via extrínseca, en el tractament amb FRT la predominant és la via intrínseca. En aquest sentit, cal tenir en compte que, si bé el mecanisme principal d'activació de caspasa 8 és a través del receptor de TNF-α, aquesta es pot activar també mitjançant la caspasa 9[80] i que la inhibició de l'activació de caspasa 8 mitjançant CsA ja s'ha vist en altres estudis[81,82].

L'any 2007, la troballa que diferents variants dels gens ATG16L i IRGM[83-85] conferien susceptibilitat per la MC va involucrar el procés d'autofàgia en la patogènia de la malaltia.

L'autofàgia té un paper molt rellevant en l'aclariment de cossos apoptòtics[86]. La persistència d'aquests cossos degut a un defecte en l'autofàgia pot contribuir a la inflamació continuada en la MC. La FRT ha demostrat eficàcia en la inducció d'autofàgia en FIH en cultiu, ja que provoca la maduració de la proteïna LC3 i l'aparició de vacuoles autofàgiques en el citoplasma d'aquestes cèl·lules. Un cop més la CsA reverteix aquest procés, i per tant inhibeix l'autofàgia induïda per FRT, demostrant que el procés d'autofàgia requereix la participació de la mitocòndria. Aquest fet indueix a pensar que l'apoptosi i l'autofàgia són dos fenòmens relacionats i que poden ser el resultat final d'una mateixa via, tal com demostren Cooney i cols.[87]. Tot i això, els nostres resultats suggereixen que l'autofàgia és independent de l'activació de les caspases ja que l'addició de z-VAD-fmk, un inhibidor universal de l'activació de les caspases, no té cap efecte sobre l'autofàgia induïda per FRT. Malgrat que els mecanismes de relació entre l'apoptosi i l'autofàgia tenen un gran interès per a la comprensió de la fisiopatologia de la MC, l'estudi d'aquests s'escapa dels objectius del present Projecte de Tesi Doctoral.

Les patologies que cursen amb desenvolupament de fibrosi estan associades a alts nivells de TGF-β que resulta en el reclutament de fibroblasts al lloc de la lesió i a un increment en la producció de MEC[88]. A nivell intracel·lular, la fosforilació d'Smad3 és el pas clau que regula les accions del TGF-β. La FRT ha mostrat eficàcia en la disminució dels nivells intracel·lulars d'Smad3 fosforilada induïda per TGF-β.

Que una molècula lipofílica interfereixi en una via intracel·lular no és sorprenent si tenim en comte estudis previs que demostren que l'α-tocoferol pot influenciar la senyalització cel·lular inhibint la proteïna cinasa C o regulant l'activació d'Akt/PKB[89-91].

Les accions de la FRT en la senyalització del TGF-β pot tenir vàries explicacions. Per una banda, la naturalesa hidrofòbica de la molècula pot determinar la seva localització en la membrana cel·lular on pot formar complexes amb les basses lipídiques ("lipid rafts"), unes estructures de la membrana que serveixen com a plataforma per a la senyalització[92]. Aquest impediment físic pot interferir en aquelles vies de senyalització que depenguin de la membrana cel·lular, com és el cas de la senyalització per TGF-β. D'altra banda, el fet que la FRT sigui capaç d'induir apoptosi i autofàgia en els FIH, pot comprometre l'estabilitat de la membrana impedint una correcta unió entre el TGF-β i el seu receptor de membrana.

La fosforilació d'Smad3 té efectes oposats en la regulació de l'expressió gènica[93]. Regula positivament l'expressió de TIMP-1, en canvi, regula negativament l'expressió de MMP-3. Tot i ser efectes oposats en quant a expressió gènica, aquests dos fenòmens afavoreixen l'acumulació de MEC. Els nostres resultats confirmen que l'estimulació de FIH amb TGF-β indueix a una sobre expressió de TIMP-1, però no em pogut demostrar que aquesta estimulació tingui un efecte inhibitori en la producció de MMP-3, segurament, això és degut a què la producció basal de MMP-3 per part dels FIH en cultiu és molt baixa.

L'exposició de FIH a FRT fa que es reverteixi aquest estat profibrogènic ja que la FRT és una potent inductora de l'expressió de MMP-3 però no afecta als nivells de TIMP-1. El paper de les MMPs en la fibrosi és controvertit i està en discussió[94,95,30]. Des del punt de vista de la inflamació, moment en què estan sobre expressades, les MMPs són responsables de la destrucció del teixit inflamat i per tant, es veuen com a proteïnes l'expressió de les quals és potencialment patològica. En canvi, en la instauració de la fibrosi, la seva expressió està fortament reprimida i es creu que l'estimulació de la seva síntesi podria ser útil en la

resolució de la fibrosi, no només pels seus efectes sobre la MEC acumulada sinó també per la seva capacitat de provocar l'apoptosi dels fibroblasts.

La síntesi de MEC i especialment la síntesi de COL1, COL3 i COL5 està incrementada en la fibrosi intestinal[96,97], aquest fet està directament relacionat amb l'activitat de TGF-β en la zona afectada. La FRT, interfereix en la síntesi de col·lagen en FIH en cultiu. Això pot tenir diverses explicacions.

Per una banda, la FRT redueix la proliferació i indueix apoptosi i autofàgia en aquestes cèl·lules, per tant, provoca una reducció en el número de fibroblasts, la qual cosa podria explicar una menor producció de MEC. Tanmateix cal tenir en comte que tot i aquests efectes en el número de cèl·lules, els FIH tractats amb FRT conserven l'habilitat de produir proteïnes, com és el cas de la MMP-3 que es troba fortament sobre expressada amb aquest tractament. Per tant, la reducció en síntesi de MEC podria no ser secundària a un procés de mort cel·lular sinó a una inhibició específica de les vies que condueixen a la seva síntesi.

D'altra banda, la FRT disminueix la fosforilació d'Smad3 induïda per TGF-β, un fet clau en la síntesi de MEC i que podria explicar els efectes de la FRT sobre la síntesi de col·lagen.

Els resultats "in vitro" discutits fins al moment permeten hipotetitzar un potencial antifibrogènic de la FRT. Per a confirmar aquest potencial "in vivo" ens calia disposar d'un model experimental de fibrosi intestinal. A diferència del que succeeix amb la colitis, en la que es disposa d'un gran nombre de models animals[98,99], hi ha pocs models experimentals específicament dissenyats per a reproduir la fibrosi intestinal. Aquest fet, ha limitat molt el desenvolupament de noves estratègies de tractament antifibrogènic en l'intestí.

Mitjançant l'administració intracolònica repetida a dosis baixes de l'haptè TNBS vam poder establir fibrosi intestinal en la rata de manera rellevant i reproduïble.

El curs temporal de la fibrosi intestinal induïda per TNBS es pot dividir en tres fases:

La **primera fase**, del dia 0 fins a dia 7, es caracteritza per una inflamació aguda que provoca pèrdua de pes, causa dany en el teixit i un infiltrat inflamatori en la zona afectada. Com a conseqüència d'aquest infiltrat hi ha un augment en el pes del colon. A més, hi ha nivells elevats de TNF-α i es dóna una important sobre expressió de MMP-3.

La **segona fase**, del dia 7 al dia 14, es caracteritza per una inflamació continuada però no progressiva. Durant aquesta fase, l'infiltrat inflamatori de la submucosa es va substituint progressivament per components cel·lulars i matriu extracel·lular. En aquesta fase hi ha un augment en l'expressió de col·lagen I i III, TNF-α i vimentina. La sobreexpressió de vimentina és molt rellevant ja que aquesta proteïna és un marcador de cèl·lules d'origen mesenquimal, com els fibroblasts i ha estat implicada en el procès de TEM, en el qual cèl·lules epitelials pateixen diversos canvis per assumir un fenotip mesenquimal.

La **tercera fase**, del dia 14 al 21, es caracteritza per una lleugera recuperació en el pes corporal de l'animal. L'infiltrat inflamatori de la submucosa colònica és completament substituït per teixit fibròtic i hi ha una marcada activació de la citoquina profibròtica TGF-β.

En aquest model de fibrosi intestinal, la FRT no ha demostrat ser capaç de prevenir o revertir els esdeveniments profibròtics que es donen al llarg de l'administració intracolònica de TNBS. Tot i això, els animals que van rebre la dosi més alta de FRT provada en aquest estudi van mostrar nivells disminuïts en paràmetres que mesuren la inflamació, això és, el tractament amb FRT va disminuir l'índex d'activitat de la malaltia i la pèrdua de pes causada pel TNBS així com també va reduir l'expressió de TNF-α.

El fet que la FRT redueixi l'expressió de TNF-α en animals tractats amb TNBS és consistent amb estudis previs que demostren un efecte antiinflamatori dels tocotrienols[100,101]. En el primer estudi els autors demostren que els tocotrienols redueixen l'expressió de TNF-α, IL-4 i IL-8 i una regulació negativa sobre NF-κB en monòcits humans induïts amb LPS. En el segon estudi els autors demostren que els tocotrienols també tenen un efecte antiinflamatori, reduint l'expressió de TNF-α, en ratolins tractats amb LPS.

Una troballa interessant de l'estudi dels efectes de la FRT en un model de fibrosi intestinal és que la dosi més alta de FRT disminueix l'expressió de vimentina en el colon dels animals tractats amb TNBS. Donat que la vimentina és una proteïna que s'expressa en quantitats elevades en fibroblasts i miofibroblasts[102], és temptador especular que aquest descens en l'expressió és degut a una disminució en el número de fibroblasts en la zona afecta com a conseqüència d'un augment en l'apoptosi d'aquestes cèl·lules. Els estudis per avaluar els nivells d'apoptosi "in situ" però no permeten aquesta afirmació, ja que hem vist que la submucosa intestinal presenta cèl·lules positives per la tinció de TUNEL tant en rates fibròtiques control

com en rates tractades amb FRT. Tot i això, aquest resultat no descarta la possibilitat de què aquesta reducció en l'expressió de vimentina sigui deguda a una disminució en el fenomen de TEM[103], ja que com hem comentat més amunt, la vimentina ha estat implicada en aquest procés.

Tot i els potents efectes antifibrogènics que la FRT té sobre FIH en cultiu, aquests no han pogut ser demostrats "in vivo" en un model de fibrosi intestinal en la rata. Això pot tenir diverses explicacions:

- El tractament de FIH amb FRT "in vitro" requereix d'un període d'incubació relativament llarg. És a dir, els efectes de la FRT sobre aquestes cèl·lules es comencen a observar passades 24-48 hores de la primera exposició. A més, la FRT demostra els seus efectes sobre FIH a concentracions molt elevades en un sistema en què la FRT està en contacte directe amb les cèl·lules, en canvi "in vivo", el contacte FRT – fibroblast intestinal pot ser molt limitat ja que requereix una absorció i distribució per tot l'animal i no podem estar segurs de quina proporció de tractament arriba realment a la zona afectada.
- La FRT no és una molècula aïllada sinó que és un extracte vegetal amb una composició complexa. A més dels tocotrienols contè coenzim Q i α-tocoferol entre d'altres. Aquest fet és molt important ja que s'ha vist que la co-suplementació de tocotrienols amb α-tocoferol disminueix l'absorció dels primers[65]. Per un estudi d'aquest tipus seria molt més convenient administrar un compost lliure d'α-tocoferol. A més, seria molt més apropiat utilitzar la molècula aïllada, concretament les isoformes gamma o delta-tocotrienol ja que són les que han demostrat un paper més potent "in vitro"[73].

CONCLUSIONS

1. L' augment en la proliferació dels fibroblasts intestinals juga un paper fonamental en la fisiopatologia de la fibrosi. El tractament *in vitro* amb FRT atenua de forma molt marcada la proliferació d'aquestes cèl·lules.
2. Com a conseqüència de l'excessiva proliferació i altres fenòmens com la migració o la TEM, transició epiteli mesènquima, es produeix una acumulació de fibroblasts en el teixit lesionat. La FRT és capaç d'induir apoptosi i autofàgia en aquestes cèl·lules, *in vitro*, reduint-ne així l'acumulació.
3. Els fibroblasts són les cèl·lules efectores en la producció de MEC, la qual s'acumula en la fibrosi. Després del tractament amb FRT, els nivells de col·lagen tipus I i laminina γ, dues proteïnes presents en la MEC, produïts per HIF disminueixen significativament.
4. La fosforilació d'Smad3 induïda per TGF-β condueix a la síntesi de MEC i a la inhibició en la degradació d'aquesta. Els fibroblasts tractats amb FRT tenen nivells inferiors d'Smad3 fosforilada després de la inducció amb TGF-β.
5. Les MMP i TIMPs són proteïnes importants en la degradació i renovació de la MEC. Els fibroblasts tractats amb FRT produeixen grans quantitats de MMP-3, en canvi, la síntesi de TIMP-1 no es veu afectada incrementant així la degradació de MEC.
6. La inflamació crònica induïda per TNBS en la rata és capaç d'instaurar una resposta fibròtica similar a la fibrosi que es dóna en la MC, fent d'aquest un model apropiat per a l'estudi dels processos que condueixen a la fibrosi intestinal i també per a l'estudi de possibles teràpies per al tractament de la fibrosi.
7. La FRT no és capaç de contrarestar la fibrosi induïda per TNBS en la rata. Tot i això sí que reverteix paràmetres indicadors d'inflamació, com la pèrdua de pes, l'índex d'activitat de la malaltia, i els nivells d'expressió de TNF-α.

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ANNEX

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Mesenchymal cell proliferation and programmed cell death: key players in fibrogenesis and new targets for therapeutic intervention

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Luna J, Masamunt MC, Lawrance IC, Sans M. Mesenchymal cell proliferation and programmed cell death: key players in fibrogenesis and new targets for therapeutic intervention. *Am J Physiol Gastrointest Liver Physiol* 300: G703–G708, 2011. First published January 13, 2011; doi:10.1152/ajpgi.00504.2010.—An exquisite equilibrium between cell proliferation and programmed cell death is re- quired to maintain physiological homeostasis. In inflammatory bowel disease, and especially in Crohn's disease, enhanced proliferation along with defective apoptosis of immune cells are considered key elements of pathogenesis. Despite the relatively limited attention that has been given to research efforts devoted to intestinal fibrosis to date, there is evidence suggesting that enhanced proliferation along with defective programmed cell death of mesenchymal cells can significantly contribute to the development of excessive fibrogenesis in many different tissues. Moreover, some therapies have demonstrated potential antifibrogenic efficacy through the regulation of mesenchymal cell proliferation and programmed cell death. Further understanding of the pathways involved in the regulation of mesenchymal cell proliferation and apoptosis is, however, required.

inflammatory bowel disease; Crohn's disease

Contribution of the Cell Proliferation/Death Equilibrium to Bowel Inflammation

It is well known that an exquisite equilibrium between cell proliferation and programmed cell death is required to maintain physiological homeostasis in any tissue. In the inflammatory bowel diseases (IBD), and especially in Crohn's disease (CD), enhanced proliferation along with defective apoptosis of immune cells are considered key pathogenic elements. This notion is supported by the histological observation that a large number of inflammatory cells infiltrate the bowel wall in patients with active IBD. Such leukocyte infiltration must result from either enhanced leukocyte recruitment from the bloodstream, an abnormal expansion of these cells within the bowel wall, or a combination of both events.

Several studies have demonstrated that T cells taken from CD patients have the ability to proliferate more than those from normal controls. Ina et al. (24) observed an increase in CD T cell proliferation upon IL-2 stimulation whereas T cells from ulcerative colitis (UC) multiplied less than control T cells. Sturm et al. (72) further expanded these observations by demonstrating that, compared with normal cells, CD T cells cycle faster, express increased phosphorylated retinoblastoma protein and decreased phosphorylated p53 levels, and undergo vigorous cellular expansion upon CD2 and CD3 stimulation. The contribution of immune cell proliferation to the development of IBD is further underlined by the efficacy shown by immunosuppressive agents such azathioprine, 6-mercaptopurine, and methotrexate in the treatment of IBD (16, 20, 56, 59, 67, 67a).

Similarly, a growing body of evidence suggests that mucosal CD T cells display an increased resistance to undergoing apoptosis. In the above mentioned study by Ina et al. (24), less apoptosis occurred in CD than control T cells upon IL-2 deprivation, a difference that could be explained by the marked decrease in the proapoptotic protein bax and an increase in the antiapoptotic protein bcl-2 found in the CD T cell population. In keeping with these results, Sturm et al. (72) showed that CD T cells display less caspase activity, but more telomerase activity, resulting in a significantly decreased rate of programmed cell death. More recently, it has been demonstrated that FAS-mediated apoptosis was lower in CD than in UC and control T cells, whereas enhanced expression of both long and short Flip (a Flice inhibitor protein) isoforms was present in both biopsy specimens and purified mucosal T cells taken from CD patients. Moreover, the authors identified that inhibition of Flip by antisense oligonucleotides could reverse the resistance of CD mucosal T cells to FAS-induced apoptosis (48). Intrinsic defects in the control of programmed cell death in mucosal T cells are strongly implicated in the pathogenesis of IBD. In addition, they may also be used to differentiate between the cellular and molecular mechanisms underlying the pathogenesis of UC and CD (57).

In the last decade several investigators have demonstrated that almost all drugs with clinical efficacy in IBD, including 5-aminosalicilates, steroids, azathioprine, methotrexate, and infliximab, are able to induce apoptosis of immune cells in vitro. This observation, combined with the fact that etanercep,

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an anti-TNF-fusion protein that does not induce apoptosis of immune cells, was not efficacious in the treatment of CD (45, 67, 71), led to two conclusions: *1*) induction of immune cells apoptosis is a key mechanism of action of many drugs useful to treat active CD patients, and *2*) when searching for new CD therapies, induction of immune cells apoptosis seems to be a requirement to be fulfilled. These concepts were broadly accepted by the IBD community until quite recently when another anti-TNF-α agent, certolizumab pegol, lacking the antibody Fc fragment and therefore unable to induce cell apoptosis, was shown to be useful for the induction and maintenance of remission in active CD (69, 70).

Therefore, the presence of abnormal immune cell proliferation and programmed cell death do contribute to CD pathogenesis and most drugs used to treat CD patients have demonstrated the ability to reverse these abnormalities, underlining the pathogenic relevance of these processes.

Contribution of Abnormal Mesenchymal Cell Proliferation to Fibrogenesis

The development of fibrosis results from an imbalance in extracellular matrix (ECM) deposition and degradation. The balance can be tipped toward a net increase in collagen and ECM production when individual cells produce a greater amount of ECM, when there are a greater number of ECM producing cells, or a combination of the two. In the presence of tissue fibrosis, there uniformly are a greater number of ECM-producing cells, which is secondary to an increase in their proliferation and a decrease in their programmed cell death.

Marked heterogeneity has been observed in the function of fibroblast-like cells between different tissues and even within the same tissue. Although it is agreed that fibrosis is almost invariably preceded by inflammation, it is unclear whether fibroblast-like cells require continuous exposure to the inflammatory microenvironment to induce fibrosis or whether a "fibrogenic" phenotype may emerge following prolonged exposure to inflammation. This was initially examined using fibroblasts isolated from idiopathic pulmonary fibrosis (IPF), a condition characterized by derangement of the alveolar wall secondary to collagen deposition. Fibroblasts from fibrosed lung tissue demonstrated markedly elevated proliferation rates in vitro when compared with those taken from normal pulmonary tissue suggesting the existence of fibroblast subgroups within pulmonary fibrosis (30). In the liver the retinoid-storing quiescent cells, in the presence of chronic inflammation, transdifferentiate into hepatic stellate cells (HSC) that display a myofibroblast phenotype and acquire contractile, proinflammatory, and fibrogenic properties (18). Similar findings are also observed in other fibrotic conditions such as scleroderma (17), urethral strictures (4), and the ECM changes associated with breast carcinoma (68).

In the intestine there also appears to be an inflammation-induced fibroblast phenotype with fibroblast-like cells isolated from IBD patients demonstrating significantly faster proliferation than those from control intestine. The proliferation rates did not differ significantly regardless of whether cells were derived from fibrosed CD, inflamed CD, and UC did not differ significantly. Similarly, following stimulation with basic fibroblast growth factor and insulin-like growth factor-1, the enhancement in proliferation was similar among the different IBD groups (32). It has also been observed that in IBD the development of intestinal fibrosis localizes to regions of active inflammation and does not differ between the type of IBD (33). This suggests that, as in the lung and liver, there is a functionally distinct subset of intestinal fibroblasts that proliferates more rapidly, is induced by chronic inflammation, and is independent of the disease type.

It seems obvious that the most effective way to prevent or limit the extent of fibrosis is to remove the underlying causative agent. This in many cases is not possible. However, if inhibition of fibroblast proliferation can be achieved, then potentially the level of ECM production and tissue fibrosis could also be reduced. Numerous agents have been suggested as potentially effective against fibrogenesis; however, data are extremely limited for most of them.

The nonsteroidal anti-inflammatory drugs (NSAID) are, as their name suggest, anti-inflammatory and block the synthesis of prostaglandins (PGs). PGE1 and 2 are known to inhibit smooth muscle cell proliferation (28) and inhibit both TNF-α- and IL-1-induced fibroblast proliferation (12). Reduced PGE2 levels are associated with the development of fibrosis in IPF (82) and the use of the NSAID indomethacin has been shown to markedly increase dermal fibrosis (43). Its use in the 2,4,6-trinitrobenzenesulfonic acid (TNBS) mouse model of intestinal fibrosis is also associated with markedly increased intestinal fibrosis (31). In IBD, PGE2 synthesis is increased in the inflamed mucosa of CD patients (2) and its levels are also increased by sulfasalazine treatment (55), but any therapeutic role that PGE2 may play in intestinal fibrosis requires further examination.

The polyunsaturated lecithin soybean extract, phosphatidylcholine (PC), has demonstrated an ability to prevent cirrhosis in a baboon model of alcohol-induced cirrhosis (35, 36), whereas it also decreased stricture formation in the rat TNBS intestinal fibrosis model (50). Benefit has also been observed with its use against tissue necrosis in immune-mediated chronic hepatitis (52) and chronic active hepatitis (9). Any role on fibroblast proliferation, however, has yet to be investigated, but polyunsaturated fatty acids like PC are precursors to PGE2, and their mechanism of action may, potentially, be through inhibition of cellular proliferation.

Another potential agent for the modification of fibrosis in IBD is the steroid hormone retinoic acid (RA). In mice its use inhibited both radiation and bleomycin-induced pulmonary fibrosis (73), whereas in humans it inhibited fibroblast proliferation in both IPF and neonatal lungs (75). In the liver it inhibits HSC proliferation (14) and dermal fibroblast proliferation both in vitro and in vivo (13). Investigation of its effect in the intestine is limited to the TNBS mouse model of intestinal fibrosis, where its use was associated with a reduction in intestinal fibrosis (31). In contrast to the above, a deficiency in RA has been associated with the development of hepatic fibrosis in the rat (82). Again, however, further work is required.

Angiotensin type 1 (AT1) receptor blockers are also potentially antifibrogenic and have been shown to attenuate liver fibrogenesis with reduction in both collagen deposition and the accumulation of myofibroblasts (49). Angiotensin II is known to induce HSC proliferation through its binding to AT1 receptors (5), whereas inhibition of angiotensin II is able to induce liver myofibroblast apoptosis (53) and reduced proliferation in

the intestinal fibroblast (41). Heparin is also known to inhibit fibroblast proliferation, as well as collagen production in human intestinal smooth muscle cells in a reversible dose-dependent manner in vitro (19). But as with all the above potentially useful agents, there is a great need for further investigation before there is enough known to recommend their clinical use.

Contribution of Abnormal Mesenchymal Programmed Cell Death to Fibrogenesis

During the perpetuation of fibrosis, mesenchymal cells activation involves discrete changes in cell behavior: proliferation, chemotaxis, contractility, matrix production, and resistance to apoptosis. It has been demonstrated that apoptosis is responsible for mediating the reduction in HSC number during the resolution of hepatic fibrosis (26) and, conversely, that induction of HSC apoptosis has an antifibrotic effect (83).

Whereas previous work has emphasized the potential importance of tissue inhibitor of metalloproteinases (TIMPs) to fibrosis via the inhibition of matrix degradation, individual TIMPs may regulate cell division and apoptosis independently of this activity. TIMP-1 suppresses HSC apoptosis both in vitro and in vivo (51), highlighting a potential role for HSC survival in liver fibrosis. So far, however, no similar work has been carried out in CD myofibroblasts.

In 1996, a susceptibility locus for CD located adjacent to the centromere on chromosome 16 was first identified (23). Further analysis of this region identified a strong association with the gene *NOD2*, also known as caspase-recruitment domain protein 15 (*CARD15*), which is involved in the recognition of bacteria with CD (80). This gene contains two amino-terminal effector domains, known as caspase-recruitment domains (CARDs), which induce the nuclear factor- B (NF- B) signaling cascade (10). The CARD domain, however, is also implicated in signal transduction that results in apoptosis via the caspases.

NOD2/CARD15 was originally shown to be expressed by monocyte/macrophage cells, but a more recent study has demon- strated expression also by intestinal myofibroblasts (54). Overex- pression of *NOD2/CARD15* enhances apoptosis through induction of caspase-9 expression. It is, therefore, attractive to speculate that mutations of this protein are implicated in the apoptotic pathway and may trigger an impaired proapoptotic response on activated cells resulting in continued activation. Indeed, a cohort study describing genotype/phenotype correlation in CD patients and *NOD2* variants showed a correlation with fibrostenosing CD (1).

In 2007, two independent genome-wide association studies (GWAS) identified *ATG16L1* as a susceptibility variant for CD (21, 63). The *ATG16L1* gene product is part of a multimeric protein complex that is essential for autophagy, a biological process that mediates the bulk degradation of cytoplasmic components in lysosomes and vacuoles. In the Wellcome Trust Case Control Consortium GWAS, a second autophagic gene was also identified with multiple SNPs in the *IRGM* gene, and this was highly associated with CD (80). It is clear from these genetic studies that autophagic processes may be associated with the pathogenesis of CD, and other studies have also demonstrated that autophagy plays an important role in clearance of apoptotic bodies (61). Persistence of apoptotic bodies as a result of incomplete autophagy could be a potential contributor to the continual inflammatory process that characterizes CD.

Until now, it was thought that *NOD2* and autophagy independently influenced the development of CD. Recently, however, studies provide a link between these two major pathways. Cooney et al. (11) identified that *NOD2* engagement by peptidoglycans induces autophagy and that this process is disturbed in individuals bearing risk alleles for either *NOD2* or *ATG16L1*, suggesting that these two genes share a common pathway. Two other polymorphisms have been also implicated in the development of fibrostenotic lesions in CD. In 2006 an association was demonstrated between T280M polymorphism of *CX3CR1* gene and fibrostenosing CD (7), whereas in 2008 V249I polymorphism of *CX3CR1* gene were also associated with fibrostenotic disease behavior (65).

CX3CR1 is a highly selective chemokine receptor for fractalkine and surface marker of NK cells, T lymphocytes, and T cells, as well as monocytes (77). The two SNPs, 249I and 280M, associated with fibrostenosis in CD are functionally relevant since they influence the binding of fractalkine to CX3CR1 (44) and result in fewer receptor binding sites and decreased ligand affinity (15, 47).

CX3CR1 is expressed on activated HSCs and, importantly, fractalkine represses TIMP-1 gene expression in these cells. The binding of fractalkine to CX3CR1-V249I, however, is associated with elevated TIMP-1 mRNA expression in hepatitis C virus-infected liver compared with CX3CR1-V (78). This effect by itself could explain the association of this allele with fibrosis given that TIMP-1 suppresses scar matrix degradation and protects HSCs from apoptosis.

Reduction in fibrosis occurs when myofibroblasts undergo apoptosis or senescence, or revert to a more quiescent phenotype, and the regulation of the balance between myofibroblasts survival vs. death may impact on the development of tissue fibrosis (58). As an example, myofibroblast apoptosis becomes evident during resolution of fibrosis and reduction in ECM content in liver cirrhosis (25) and renal fibrosis (3), suggesting a role for myofibroblast apoptosis in the resolution of tissue fibrosis.

Previous studies have demonstrated that hepatocyte growth factor (HGF) reduces lung fibrosis in murine models (76, 84) and there is ample evidence that HGF plays an essential part in parenchymal repair and protection in other organs (6, 42). It has been suggested that HGF is a potent inducer of ECM-degrading enzymes such as the matrix metalloproteinases (MMPs) (42), which are overexpressed during myofibroblasts apoptosis (25). MMPs induce apoptosis in lung myofibroblasts through the extracellular degradation of fibronectin and that the antifibrotic effects of HGF observed in lung were due to upregulation of MMPs and MMP-dependent myofibroblast apoptosis (46).

In that regard, a variety of dietary components, including vitamin E, have attracted attention for their health benefit and harmless consumption profile. Specifically, tocotrienols, which have proven efficacy in inducing apoptosis and autophagy on activated rat pancreatic stellate cells (PSC) and human intestinal fibroblasts (HIFs) (62, 40). Tocotrienols are able to induce apoptosis in activated fibroblasts by activating caspase 3, 8, and 9 and increasing DNA fragmentation. Furthermore, upon treatment with tocotrienols, both PSCs and HIFs display an autophagic response by converting LC3I to LC3II. Impor-

tantly, tocotrienols have no such effects in quiescent PSCs and acinar cells from the pancreas, showing selectivity to activated cells. Interestingly, tocotrienols are also able to upregulate expression of MMPs on HIFs in vitro (39), which, besides inducing accumulated ECM degradation, could be responsible for the induction of fibroblast apoptosis observed upon tocotrienol treatment.

NF-κB signaling promotes survival of hepatic myofibroblasts (79). Angiotensin II, which is locally synthesized in the injured liver promotes HSC proliferation (5), myofibroblast survival and liver fibrosis through the activation of NF-κB (53). Losartan, an angiotensin II type I receptor, has some efficacy in attenuating liver fibrosis (49, 86) and triggers apoptotic cell death in human pancreatic cancer (60) and stellate cells (37). The angiotensin-converting enzyme inhibitor captopril can also prevent fibrosis development in experimental colitis in the rat (81) and attenuates the progression of rat hepatic fibrosis (29). No studies, however, have been carried out to assess its apoptotic effect on fibroblasts, but it does induce apoptosis in other cell types, including human vascular myocytes (8) and vascular smooth muscle cells (22).

Anti-inflammatory and antifibrotic effects of the widely used cholesterol level-lowering 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (statins) have been also examined in several in vitro models. Statins may be effective antifibrotic agents through inhibition of the activation and proliferation of fibrogenic cells and ECM production (27, 34, 38, 64, 66). Lovastatin is able to induce lung fibroblasts apoptosis (74) and pravastatin induces apoptosis of HSC (85). Of the antifibrotic mechanisms of the statins, induction of activated fibroblasts apoptosis appears be the most important. Fibrosis has been considered traditionally as an irreversible process but experimental and clinical literature data published in the last decade have suggested that an effective therapy can result in significant regression of fibrosis. This is usually associated with induction of apoptosis of mesenchymal cells.

Conclusions

Despite the limited attention that has been given to research efforts devoted to intestinal fibrosis to date, there is evidence that suggests that enhanced proliferation along with defective programmed cell death of mesenchymal cells can significantly contribute to the development of abnormal fibrogenesis in many different tissues. In line with these findings, there are a few therapies that have demonstrated potential antifibrogenic efficacy through the regulation of mesenchymal cell proliferation and programmed cell death. Further understanding of the pathways involved in the regulation of mesenchymal cell proliferation and apoptosis, as well as further evaluation of the potentially antifibrogenic agent, is required before there is going to be effective therapy directed against intestinal fibrosis.

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DISCLOSURES

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